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<b>(54) Title:</b> A COMPOSITION FOR THE PREVENTION AND/OR TREATMENT OF ATHEROSCLEROSIS  <b>(57) Abstract</b>  An immunological oral tolerance-inducing composition for prevention and/or treatment of atherosclerosis, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta <sub>2</sub> -glycoprotein-1(β <sub>2</sub> GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.		

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## **A COMPOSITION FOR THE PREVENTION AND/OR TREATMENT OF ATHEROSCLEROSIS**

### **Technical Field**

The present invention relates to an immunological oral tolerance-inducing composition for the prevention and/or treatment of atherosclerosis.

### **Background Art**

Atherosclerosis and its clinical sequelae represent one of the most hazardous insults on Western society in terms of morbidity and mortality. Hence, it is not surprising that immense efforts have been put forward to investigate its etiopathogenesis aiming to provide means of slowing or halting its early indolent progression. Although no definite answers exist it is becoming apparent that not one individual mechanism is solely responsible for the evolvement of the atherosclerotic plaque as was formerly presumed with regard to dysregulation of the lipid balance.

Clinicians use the expression atherosclerosis to define a histopathological process which results in occlusion of arteries in different parts of the body. The occlusion is caused by the accumulation of different cells of various origins which are gradually being filled with large amounts of fat from the circulation. The fat which mainly consists of 'bad' cholesterol low density lipoprotein (LDL), is the principal material which deposits in the vessel walls. In the later stages of atherosclerosis, the lipid filled zones begin to accumulate calcium. The deposition of calcium renders the arteries stiffer and less flexible, thereby causing their 'stony' appearance, an appearance which has led to the well-known expression of "sclerosis of the arteries" (arteriosclerosis). When the involved arteries block the blood flow to the heart, a person is afflicted with a 'heart attack'; when the brain arteries occlude, the person experiences a stroke. When arteries to the limbs narrow, the result is severe pain, decreased physical mobility and possibly the need for an amputation.

It is clear that the progression of the above-mentioned diseases is non-detectable for many years and only manifests themselves at advanced stages. When these manifestations become detectable it is much more difficult to offer a remedy, due to the advanced stages of the disease.

As mentioned above, atherosclerosis is the result of the deposit of fat in the walls of blood vessels, thereby creating a layer of atherosclerotic plaque, said layer

clogs the flow of blood to vital organs, thereby leading to cerebrovascular accidents, myocardial infarctions or peripheral blood vessel diseases. Known risk factors for rapid progression of the condition include high blood pressure, diabetes, overweight, smoking and the lack of physical exercise.

In recent studies it appears that infectious factors such as Cytomegalo virus (CMV), Epstein-Bar (EBV) and Chlamydia pneumonia may also be involved in the progression of atherosclerosis. The above has been demonstrated by linking atherosclerosis with gingivitis, in addition to the correlation between vessel obstruction and restenosis and the titers of autoantibodies to anti-CMV antigens, or finding infectious particles in the atherosclerotic plaque.

In the last decade a body of evidence has been accumulated to support the theory that atherosclerosis has a significant infectious-autoimmune component.

In autoimmune diseases the immune system attacks our body components (autoantigens), in addition to attacking external invaders. The autoimmune diseases are classified as autoantibody mediated or cell mediated diseases. Typical autoantibody mediated autoimmune diseases are myasthenia gravis and idiopathic thrombocytopenic purpura (ITP), while typical cell mediated diseases are Hashimoto's thyroiditis and Diabetes type I.

#### **Involvement of the immune network in atherosclerosis**

The recognition that immune mediated processes prevail within atherosclerotic lesions stemmed from the consistent observation of lymphocytes and macrophages in the earliest stages, namely the fatty streaks. These lymphocytes which include a predominant population of CD4+ cells (the remainder being CD8+cells) were found to be more abundant over macrophages in early lesions, as compared with the more advanced lesions, in which this ratio tends to reverse. These findings posed questions as to whether they reflect a primary immune sensitization to a possible antigen or alternatively stand as a mere epiphenomenon of a previously induced local tissue damage. Regardless of the factors responsible for the recruitment of these inflammatory cells to the early plaque they seem to exhibit an activated state manifested by concomitant expression of MHC class II HLA-DR and interleukin (IL) receptor as well as leukocyte common antigen (CD45R0) and the very late antigen 1 (VLA-1) integrin.

The on-going inflammatory reaction in the early stages of the atherosclerotic

lesion may either be the primary initiating event leading to the production of various cytokines by the local cells (i.e endothelial cells, macrophages, smooth muscle cells and inflammatory cells), or it may be that this reaction is a form of the body's defense immune system towards the hazardous process. The cytokines which have been shown to be upregulated by the resident cells include TNF- $\alpha$ , IL-1, IL-2, IL-6, IL-8, IFN- $\gamma$  and monocyte chemoattractant peptide-1 (MCP-1). Platelet derived growth factor (PDGF) which is expressed by all cellular constituents within atherosclerotic plaques have also been shown to be overexpressed, thus possibly intensifying the preexisting inflammatory reaction by a co-stimulatory support in the form of a mitogenic and chemotactic factor. Very recently, Uyemura K, Demer LL, Castle SC et al. Cross regulatory roles of IL-12 and IL-10 in atherosclerosis. *J Clin Invest* 1996 97; 2130-2138 have elucidated type 1 T-cell cytokine pattern in human atherosclerotic lesions exemplified by a strong expression of IFN- $\gamma$  but not IL-4 mRNA in comparison with normal arteries. Furthermore, IL-12 - a T-cell growth factor produced primarily by activated monocytes and a selective inducer of Th1 cytokine pattern, was found to be overexpressed within lesions as manifested by the abundance of its major heterodimer form p70 and p40 (its dominant inducible protein) mRNA.

Similar to the strong evidence for the dominance of the cellular immune system within the atherosclerotic plaque, there is also ample data supporting the involvement of the local humoral immune system. Thus, deposition of immunoglobulins and complement components have been shown in the plaques in addition to the enhanced expression of the C3b and C3Bi receptors in resident macrophages.

Valuable clues with regard to the contribution of immune mediated inflammation to the progression of atherosclerosis comes from animal models. Hence, it seems that immunocompromised mice (class I MHC deficient ) tend to develop accelerated atherosclerosis as compared with immune competent mice. Additionally, treatment of C57BL/6 mice (Emeson EE, Shen ML. Accelerated atherosclerosis in hyperlipidemic C57BL/6 mice treated with cyclosporine A. *Am J Pathol* 1993; 142: 1906-1915) and New-Zealand White rabbits (Roselaar SE, Schonfeld G, Daugherty A. Enhanced development of atherosclerosis in cholesterol fed rabbits by suppression of cell mediated immunity. *J Clin Invest* 1995; 96:

1389-1394) with cyclosporine A, which is- a potent suppressor of IL-2 transcription resulted in a significantly enhanced atherosclerosis under "normal" lipoprotein "burden". These latter studies may provide insight into the possible roles of the immune system as engaged in counteracting the self-perpetuating inflammatory process within the atherosclerotic plaque.

Atherosclerosis is not a classical autoimmune disease, although some of its manifestations such as the production of the plaque which obstruct the blood vessels may be related to immune system effects. In classical autoimmune disease, one can also define very clearly the autoantigen attacked by the immune system, one can define the part of the immune system which attacks the autoantigen (autoantibody or cells) which belong to the immune system and are named lymphocytes. Above all one can show that by passive transfer of these components of the immune system the disease can be induced in healthy animals, or in the case of humans the disease may be transferred from a sick pregnant mother to her offspring. Many of the above are not prevailing in atherosclerosis. In addition, the disease definitely has common risk factors such as hypertension, diabetes, lack of physical activity, smoking and others, the disease affects elderly people and has a different genetic preponderance than in classical autoimmune diseases.

The process of inducing oral tolerance has been known since the beginning of the century for reducing allergic reactions. The allergic patient was fed with a low dose of the known allergen and the body's immune tolerance was restored and no allergic reaction occurred.

#### ***Oral tolerance in autoimmune diseases***

In autoimmune diseases oral tolerance is a term denoted to describe blunting the immune response in an autoimmune disease. The tolerization produced by feeding an animal with the 'incriminated' antigen, can abrogate the development of the disease due to a 'paralyzing' effect on the immune response. The present inventors have recently shown that oral feeding with human  $\beta$ 2GPI prior to immunization with this molecule, resulted in amelioration of the anti-phospholipid (APS)-equivalent syndrome.

Oral tolerance has been applied in the realm of autoimmune diseases, in which an immune reaction was carried out against the autoantigen and there existed a need for restoring the immune system's tolerance to the autoantigen.

This was carried out by feeding a subject with low doses of the autoantigen. So far, several animal models have been reported in which oral tolerance has been restored, including preventing the development of experimental allergic encephalomyelitis (EAE), which is the equivalent of multiple sclerosis (MS), by feeding a subject with a protein from the nerve membrane called myelin basic protein (MBP), in addition to preventing adjuvant arthritis and collagen arthritis, by feeding a subject with collagen and HSP-65, respectively. A Boston based company called Autoimmune has carried out several human experiments for preventing diabetes, multiple sclerosis, rheumatoid arthritis and uveitis. The results of the human experiments have been less impressive than the non-human ones, however there has been a success with the prevention of arthritis.

## **Antigenic Components**

### ***A. Oxidized low density lipoprotein***

LDL is a complex of large molecular weight proteins, including apolipoprotein B, neutral and polar lipids and lipophilic antioxidants (vitamin E and  $\beta$  carotene). The oxidation-modification of LDL with the resultant formation of neopeptides within the native molecule (within the Apo B domain) leads to its recognition by the scavenger receptor on the macrophages. It is possible that all cellular components in the atherosclerotic plaque (i.e. endothelial cells, macrophages, smooth muscle cells, lymphocytes) are capable of enhancing lipid peroxidation with the production of varying degrees of LDL oxidation, yet the relative contribution of each, has not been determined. Regardless of the precise roles of these cells it appears that the balance between the oxidant and antioxidant forces at the level of the vessel walls determines the extent of exposure to Ox LDL with the subsequent deleterious effects. Ox LDL has active derivatives such as lysophosphatidylcholine (LPC) and, despite having a smaller molecular weight, still retains some of its biological activities.

Lysophosphatidylcholine (LPC) is expressed in human atherosclerotic plaques. It is an active biological substance that can induce the first steps of atherogenesis. Indeed it is even more potent than Ox LDL.

The overall *in-vivo* and *in-vitro* influence of Ox LDL and its byproducts on the development of the plaque are well beyond the scope of the present invention,

however it is important to state the known relationship between Ox LDL and the immune system.

It is known that Ox LDL is chemotactic for T-cells and monocytes. Ox LDL and its byproducts are also known to induce the expression of factors such as monocyte chemotactic factor 1, secretion of colony stimulating factor and platelet activating properties, all of which are potent growth stimulants.

The active involvement of the cellular immune response in atherosclerosis has recently been substantiated by Stemme S, Faber B, Holm J. T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein. Proc Natl Acad Sci USA 1995; 92: 3893-97, who isolated CD4+ within plaques clones responding to Ox LDL as stimuli. The clones corresponding to Ox LDL (4 out of 27) produced principally interferon- $\gamma$  rather than IL-4. It remains to be seen whether the above T-cell clones represent mere contact with the cellular immune system with the inciting strong immunogen (Ox LDL) or that this reaction provides means of combating the apparently indolent atherosclerotic process.

The data regarding the involvement of the humoral mechanisms and their meaning are much more controversial. Several reports have associated increased levels of antibodies to Ox LDL with the progression of atherosclerosis (expressed by the degree of carotid stenosis, severity of peripheral vascular disease etc.). However, these data were not reproduced by other scientists, perhaps due to the lack of standardization with regard to the assays used for determining antibodies to LDL. In any case there seems to be a consensus as to the presence of Ox LDL antibodies in the form of immune complexes within atherosclerotic plaque, although the true significance of this finding has not been established.

Antibodies to Ox LDL have been hypothesized as playing an active role in lipoprotein metabolism. Thus, it is known that immune complexes of Ox LDL and its corresponding antibodies are taken up more efficiently by macrophages in suspension as compared with Ox LDL. No conclusions can be drawn from this consistent finding on the pathogenesis of atherosclerosis since the question of whether the accelerated uptake of Ox LDL by the macrophages is beneficial or deleterious has not yet been resolved.

Important data as to the significance of the humoral immune system in atherogenesis comes from animal models. It has been found that



hyperimmunization of LDL-receptor deficient rabbits with homologous oxidized LDL, resulted in the production of high levels of anti-Ox LDL antibodies and was associated with a significant reduction in the extent of atherosclerotic lesions as compared with a control group hyperimmunized with phosphate-buffered saline (PBS). A decrease in plaque formation has also been accomplished by immunization of rabbits with cholesterol rich liposomes with the concomitant production of anti-cholesterol antibodies, yet this effect was accompanied by a 35% reduction in very low density lipoprotein cholesterol levels. The present inventors have shown that in apoE knockout mice, repeated immunizations with homologous Ox LDL results in production of anti-Ox LDL antibodies and in reduced atherosclerosis.

#### ***B. Heat Shock protein (HSP) 60/65***

An additional major antigenic component for the initiation and perpetuation of the inflammatory lesion culminating in enhanced atherosclerosis is the 60 Kd heat shock protein. This mitochondrial protein is a member of the HSP family which constitutes about 24 proteins displaying high degree of sequence homologies between different species. These proteins, as their name implies are upregulated in response to various stressful insults including exposure to free radicals, heat, mechanical shear stress, infections, cytokines ect. The teleological importance of the HSP stems from their protective roles against unfolding of cellular proteins precipitated by stressful stimuli. This role has led to their designation as molecular 'chaperones'. However, these apparently favorable effects of HSP's can be a double-edged sword, since their overexpression may, under certain conditions promote an autoimmune reaction with resultant tissue damage. The mechanisms responsible for the HSP immune mediated damage are not completely understood and it is presumed that cryptic neoepitopes are exposed to the immune system which no longer consider them as 'self' following their upregulation. Alternatively, it was suggested that cross reaction exists between 'foreign' HSP and self HSP introduced following infections which act to trigger an immune attack against self structures.

HSP 60 is a distinct protein which constitutes a separate "class" within the above-mentioned HSP family, together with HSP 65, providing a sequestered environment for folding a subset of proteins in-vivo.

There exists a similarity between mammalian HSP60 and bacterial HSP65, which allows for cross recognition by immune effectors of the host.

Support for the involvement of HSP in autoimmunity is provided by studies documenting enhanced autoantibody as well as cellular response to HSP 60/65 in several autoimmune diseases.

The link between HSP 65 and atherosclerosis was initially raised following a pioneering work conducted by Georg Wick's group in the early 1990s (Xu Q, Dietrich H, Steiner HJ & al. Induction of arteriosclerosis in normocholesterolemic mice rabbits by immunization with heat shock protein 65. *Arteriosclerosis Thrombosis* 1992; 12: 789-799). They found that rabbits immunized with different antigens developed atherosclerosis, provided the preparation used for immunization contained complete Freund's adjuvant (CFA). Since the major constituent of CFA is heat killed mycobacterium tuberculosis the principal component of which is the HSP-65, they reasoned that the immune response towards this component led to the development of atherosclerosis. This hypothesis was confirmed when the animals were subsequently immunized merely with HSP-65 and exhibited pronounced atherosclerosis. It should be emphasized that no difference was noted between the groups with regard to the lipoprotein profile. Additional work conducted later by Wick's group revealed that T cells extracted from lesions of the rabbits induced to develop atherosclerosis were found to overexpress HSP-65 in comparison with peripheral blood from the respective animals thus attesting for a localized and restricted immune reaction in the vicinity of the stressed arterial vessel. The present inventors have reinforced Wick's finding, by showing that HSP-65 (or *Mycobacterium tuberculosis*) immunization of naive mice resulted in accelerated fatty streak formation.

These findings were further substantiated when involvement of humoral immune mechanisms in response to HSP-65 were observed in patients inflicted with atherosclerosis. Hence, a correlation has been established between high levels of anti-HSP65 antibodies and the extent of sonographically estimated carotid narrowing in a screen of healthy individuals.

These findings were recently reinforced by *in-vitro* assays designed to assess the influence of HSP-60/65 on cultured endothelial cells. Accordingly, it has been shown that incubation of endothelial cells with HSP65 induced their

adhesiveness to monocytes and granulocytes which was concentration and time dependent. Furthermore, it has been demonstrated that this effect is mediated by overexpression of endothelial cells E-selectin (C62E), vascular cell adhesion molecule-1 (CD106) and intracellular adhesion molecule-1 (CD54).

***Phospholipids and  $\beta$ 2GPI as autoantigen components in atherosclerosis.***

The present inventors have found that the production of anticardiolipin in LDL-receptor knockout mice by immunization with human anticardiolipin antibodies (aCL) results in accelerated early atherosclerosis. This observation is consistent with circumstantial data that has accumulated with regard to the possible proatherogenic role of anticardiolipin antibodies in atherosclerosis.

$\beta$ 2GPI is a molecule that was proved as the target of aCL from patients with autoimmune diseases. Anti- $\beta$ 2GPI antibodies have been shown to result in experimental APS and to activate endothelial cells and platelets. The present inventors have shown that transgenic LDL-RD deficient mice immunized with  $\beta$ 2GPI develop respective antibodies and that early fatty streak formation is enhanced, accompanied by local accumulation of CD4+ cells. Thus,  $\beta$ 2GPI may serve as an autoantigenic target of the autoimmune response in atherosclerosis.

In both animal and human disease models a correlation has been found between the titer autoantibodies to the autoantigens HSP 60-65 and Ox LDL and the level of atherosclerosis. The Ox LDL is produced in the body by the oxidation of the native LDL and is considered the noxious factor of atherosclerosis. In response to said toxin, anti-Ox LDL antibodies are produced. The  $\beta$ 2GP-I is a natural protein in human blood which plays a role in the coagulation process and HSP65 is the result of various stress stimuli.

In experiments carried out with mice having a predisposition for the development of atherosclerosis (LDL-R knock-out mice), a thirty percent reduction was demonstrated in the atherosclerosis condition by feeding the mice with low doses of Ox LDL.

Furthermore, the fact that the immune system is involved in the induction of atherosclerosis, as well as the clearcut involvement of the immune system (lymphocytes and autoantibodies), points to the ability to manipulate the disease by inducing oral tolerance with autoantigens, e.g., Ox LDL, HSP60/65 and  $\beta$ 2GPI.

U.S. Patent No. 5,348,945 discloses a method of combating mortality in a cell or tissue under stress. The method comprises contacting heat shock protein 70 (HSP70) to the cell or tissue in an amount effective to enhance the survival of that cell or tissue. The method may be employed in the combating of atherosclerosis, restenosis after angioplasty and nerve damage in human or animal subjects in need of such treatment. A pharmaceutical composition comprising a therapeutically effective amount of HSP70 in a pharmaceutically acceptable formulation is also disclosed.

Although HSP70 and HSP60 belong to a family of about 24 highly conserved heat shock proteins, they represent two entirely distinct characteristics. Their mechanism, for example, do not appear to act in concert in governing the protection from stressful stimuli.

HSP70 was initially patented because of its pronounced induction during heat exposure and other stressful insults such as ischemic preconditioning. Indeed the overexpression of HSP70 in transgenic animals is associated with protection from stressful hazards.

Therefore, U.S. Patent No. 5,348,945 does not teach or suggest the subject matter of the present invention.

#### **Disclosure of the Invention**

Thus, according to the present invention there is now provided an immunological oral tolerance-inducing composition for prevention and/or treatment of atherosclerosis, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In an preferred embodiment of the present invention there is provided an immunological oral tolerance-inducing composition for prevention and/or treatment of a heart attack, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In another preferred embodiment of the present invention there is provided an immunological oral tolerance-inducing composition for prevention and/or treatment of angioplasty-restenosis, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In a further preferred embodiment of the present invention there is provided an immunological oral tolerance-inducing composition for prevention and/or treatment of stroke, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In even further preferred embodiments of the present invention there is provided an immunological oral tolerance-inducing composition wherein said active component is a modified low-density lipoprotein, or wherein said active component is oxidized low-density lipoprotein (Ox LDL), or wherein said active component is an active derivative of oxidized low-density lipoprotein (Ox LDL), or wherein said active component is heat shock protein 60/65 (HSP 60/65), or wherein said active component is beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1).

The present invention provides an immunological oral tolerance-inducing composition, wherein said active derivative is lysophosphatidyl choline (LPC).

The present invention also provides an immunological oral tolerance-inducing composition, wherein said LDL is malondialdehyde LDL (MDA-LDL).

In another aspect of the present invention there is provided a method for prevention and/or treatment of atherosclerosis in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1), functional derivatives thereof and

mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In a preferred embodiment there is provided a method for prevention and/or treatment of a heart attack in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In a further preferred embodiment there is provided a method for prevention and/or treatment of angioplasty-restenosis following angioplasty in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In an even further preferred embodiment there is provided a method for prevention and/or treatment of stroke in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In further embodiments of the present invention there is provided a method for prevention and/or treatment of atherosclerosis in a subject, wherein said active component is a modified low-density lipoprotein, or wherein said active component is oxidized low-density lipoprotein (Ox LDL), or wherein said active component is an active derivative of oxidized low-density lipoprotein (Ox LDL), or wherein said active component is heat shock protein 60/65 (HSP 60/65), or wherein said active component is beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1).

The present invention further provides a method prevention and/or treatment of atherosclerosis in a subject, wherein said active derivative is lysophosphatidyl choline (LPC).

The present invention further provides a method prevention and/or treatment of atherosclerosis in a subject, wherein said LDL is malondialdehyde LDL (MDA-LDL).

The term "functional derivative" as used herein is intended to include labelled proteins, conjugated proteins, fused chimeric proteins and purified receptors in soluble form, as well as fragments, deletions, and conservative substitutions of said proteins.

The existence of an immune response against Ox LDL in atherosclerosis and the correlation between the reaction to Ox LDL and the severity of the disease, in combination with evidence that an active vaccine of Ox LDL in mice and rabbits can prevent the development of atherosclerosis has led the present inventors to conclude that the induction of immune tolerance by feeding Ox LDL to a human subject can result in the reduced rate of atherosclerosis progression. It should be mentioned that the mechanisms of inducing immune tolerance by mouth feeding are possibly mediated via a stimulation and production of cytokine TGF $\beta$  and the development of non-specific suppresser T-cells.

The oral tolerization of the present invention may extend to yield a bystandard suppression effect: namely - blocking other (non-antigen specific) autoimmune (anti-self) responses occurring in the vicinity of the atherosclerotic plaque and contributing to its progression.

It should be noted that the aim of the present invention is to induce tolerization or paralyze the immune response towards the HSP65, rather than to achieve mere elevation in the serum to assist protein unfolding.

Therefore, in one aspect the present invention combines oral tolerance, Ox LDL and atherosclerosis, which is a disease caused in part by immune factors. Ox LDL has been reported to induce an immune reaction in mice and rabbits (in contrast to inducing immune tolerance) of Ox LDL antigens and an improvement in the atherosclerosis condition. In these animal models Ox LDL has not been reported to have been experimented with mouth feeding and has never been suggested for oral tolerance.

The pharmaceutical compositions for oral administration according to the present invention are prepared by methods known per se and the administration thereof is by known methods of oral administration.

An amount effective to treat the disorder hereinbefore described depends on the usual factors such as the nature and severity thereof and the weight of the mammal.

For oral administration, it is preferred that the active ingredient be administered in the form of a unit-dose composition.

Such compositions are prepared by admixture and are suitably adapted for oral administration in the form of tablets, capsules, oral liquid preparations, powders, granules, etc.

Tablets and capsules for oral administration are usually presented in a unit dose, and contain conventional excipients such as binding agents, fillers, diluents, tableting agents, lubricants, disintegrants, colorants, flavorings, and wetting agent. The tablets may be coated according to well known methods in the art.

Suitable fillers for use include cellulose, mannitol, lactose and other similar agents. Suitable disintegrants include starch, polyvinylpyrrolidone and starch derivatives such as sodium starch glycolate. Suitable lubricants include, for example, magnesium stearate. Suitable pharmaceutically acceptable wetting agents include sodium lauryl sulphate.

These solid oral compositions may be prepared by conventional methods of blending, filling or tableting. Repeated blending operations may be used to distribute the active agent throughout those compositions employing large quantities of fillers. Such operations are, of course, conventional in the art.

Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups, or elixirs, or may be presented as a dry product for reconstitution with water or with another suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, for example, sorbitol, syrup, methyl cellulose, gelatin, hydroxyethylcellulose, carboxymethyl cellulose, aluminium stearate gel or hydrogenated edible fats, emulsifying agents, for example lecithin, sorbitan monooleate, or acacia; non-aqueous vehicles (which may include edible oils), for example, almond oil, fractionated coconut oil, oily esters such as esters of glycerine, propylene glycol, or



ethyl alcohol; preservatives, for example methyl or propyl p-hydroxybenzoate or sorbic acid, and if desired conventional flavoring or coloring agents.

Oral formulations also include conventional sustained release formulations, such as tablets or granules having an enteric coating.

### **Description of Preferred Embodiments**

While the invention will now be described in connection with certain preferred embodiments in the following examples so that aspects thereof may be more fully understood and appreciated, it is not intended to limit the invention to these particular embodiments. On the contrary, it is intended to cover all alternatives, modifications and equivalents as may be included within the scope of the invention as defined by the appended claims. Thus, the following examples which include preferred embodiments will serve to illustrate the practice of this invention, it being understood that the particulars shown are by way of example and for purposes of illustrative discussion of preferred embodiments of the present invention only and are presented in the cause of providing what is believed to be the most useful and readily understood description of formulation procedures as well as of the principles and conceptual aspects of the invention.

### **MATERIALS AND METHODS**

#### ***Animals.***

Twelve weeks old LDL-RD mice (hybrids of between the C57BL/6J and 129Sv strains) are created by homologous recombination. The mice are purchased from The Jackson Laboratory (Bar-Harbor ME). The LDL-RD mice are used since they have high cholesterol levels on chow diet (which are similar to human values) and develop significant atherosclerosis only when fed a high fat diet. The LDL-RD mice are either fed with a normal chow-diet containing 4.5% fat by weight (0.02% cholesterol) or an atherogenic diet (Harlan, Teklad Premier Laboratory Diets, Madison, WI) containing: 1.25% cholesterol, 7.5% casein and 0.5%(wt) sodium-cholate. The mice are maintained on 12 hour-dark/12 hour light cycles and are allowed to access food and water *ad libitum*.

#### ***LDL isolation, oxidation and characterization***

Blood for lipoprotein isolation is collected in EDTA (1mg/ml) after 12 hours of fasting. LDL (density=1.019-1.063 g/l) is isolated from the plasma after density adjustment with KBr<sup>-</sup>, by preparative ultracentrifugation at 50,000 rpm/min for 22

hours, using type 50 rotor. The LDL preparations are washed by ultracentrifugation, dialyzed against a pH 7.4, 0.15 mol/L EDTA, passed through Acrodisc filter (0.22  $\mu$ m pore size) to remove aggregates, and stored under nitrogen in the dark.

LDL oxidation is performed by incubation of pre-dialyzed LDL (1 mg of protein/ml in EDTA-free PBS) with copper sulfate (10  $\mu$ M) for 24 h at 37° C. Lipoprotein oxidation is confirmed by analysis of thiobarbituric acid-reactive substances (TBARS) which measures malondialdehyde (MDA) equivalents by the lipid peroxidation test and also by analysis of the conjugated diene content of the lipoprotein.

***Determination of anti-Ox LDL antibody titers.***

Preparation of native and copper-oxidized LDL is preformed. Ninety six well polystyrene plates (Nunc Maxisorp, Denmark) are coated with either Ox LDL, native LDL (at concentration of 5  $\mu$ g/ml in PBS) or PBS overnight at 4°C. After washing four times with PBS containing 0.05% Tween and 0.001% aprotinin (Sigma, USA) the plates are blocked with 2% bovine serum albumin (BSA) for 2 hours at room temperature. Serum fractions are diluted to 1:50 in PBS 0.05% Tween 0.2% BSA. After an additional overnight incubation the plates are washed and alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch laboratory Inc., USA) are added and diluted to 1:10,000 in PBS 0.05% Tween-0.2% BSA for 1 hour at room temperature. After extensive washing, 1 mg/ml *p*-nitrophenyl-phosphate (Sigma) in 50 mM carbonate buffer containing 1mM MgCl<sub>2</sub> pH 9.8 is added as a substrate. The reaction is stopped after 30 min by adding 1 M of NaOH. The color is read at a 405 nm wavelength in a Titertek ELISA reader (S.L.T Laboratory Instruments, Vienna, Austria) and results expressed as O.D. at 405 nm. The titer of anti-Ox LDL antibodies is calculated by subtracting the value obtained from binding to native LDL from the binding to Ox LDL.

Inhibition assays are performed to confirm the specificity of anti-Ox LDL antibodies and to check for a possible cross-reactivity with HSP-an important immunogen in atherosclerosis. The concentration of HSP-65-immunized mouse serum giving half of the maximal binding to Ox LDL are determined and different inhibitors (i.e. HSP-65, Ox LDL, bovine serum albumin) are applied in increasing concentrations.

***Detection of Anti-HSP-65 Antibodies.***

Recombinant HSP-65 (1 $\mu$ g/ml) in phosphate buffered saline (PBS, pH 7.2) are coated onto flat bottom 96-well ELISA plates (Nunc Maxisorp, Denmark) by overnight incubation at 4°C. After washings with 0.02% PBS Tween and blocking with 1% BSA in PBS, sera are added in different dilutions (1:50, 1:100, 1:200, in PBS) and incubated for 1 hr at room temperature. Peroxidase conjugated rabbit anti-mouse IgG (Dako Ltd, High Wycombe, UK) are added and incubated for 1 hr at room temperature followed by 4 washings with PBS/Tween. Finally, 100 $\mu$ l of citrate phosphate buffer (0.1 M, pH 4.2) containing 0.53 mg/ml of 2,2-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Sigma) are added, and absorbance is measured after 30 min at 490 nm in a Titertek ELISA reader.

***Detection of anti-phospholipid antibodies.***

A modified ELISA to determine reactivity of the mouse and human antibodies to PL is performed. Microtiter plates (Nunc, Maxisorp, Denmark) are coated either with an anionic PL [cardiolipin (CL), phosphatidylserine (PS), phosphatidylinositol (PI)] and phosphatidylcholine (PC), all from Sigma Chemicals Co. St. Louis, Mo, at a concentration of 50  $\mu$ g/ml and dissolved in ethanol, except for PC (chloroform-methanol, 1: 3). Plates are dried under vacuum and blocked with TBS containing 0.5% gelatin. The plates are then washed three times with TBS and different concentrations of mouse preparations are added to the wells treated with human  $\beta$ 2GPI (5 $\mu$ g/ml for 30 min) or 0.1% gelatin/TBS alone. The binding of the antibodies is detected with goat anti-mouse alkaline phosphatase conjugate and the addition of substrate (*p*-nitro-phenylphosphate). The results are expressed as absorbance at 405 nm (OD<sub>405</sub>).

***Detection of anti- $\beta$ 2GPI antibodies.***

One  $\mu$ g/ml  $\beta$ 2GPI or a different DM is incubated overnight in 50 mM bicarbonate buffer, pH 9.6 at 4°C in 96-well polystyrene plates (Nunc). After 3 washings with TBS, blocking is performed with 0.5% gelatin/TBS for 2 hrs (as for the anti-PL ELISA). The plates are then washed three times and murine sera (diluted in 1: 100 in gelatin/TBS) is added and incubated for 2 hrs at room temperature. After 3 washings, alkaline phosphatase conjugated goat anti-human IgG respectively diluted in 0.1% gelatin/TBS (1:10,000) is added for 2 hrs. After 3

additional washings, the substrate *p*-nitrophenylphosphate in a sodium carbonate buffer pH 9.8 is added and absorbance is read at 405 nm.

#### ***Proliferation Assays of Spleen Lymphocytes.***

Spleens are removed from mice upon sacrifice, and lymphocytes are isolated.  $1 \times 10^6$  cells per ml are incubated in triplicates for 72 h in 0.2 ml of culture medium in microtiter wells in the presence of different concentrations of HSP-65, Ox LDL,  $\beta$ 2GPI or ovalbumin. Proliferation is measured by the incorporation of [ $^3$ H] thymidine into DNA during the final 12 hours of incubation. The results are computed as stimulation index (S.I.): the ratio of the mean cpm of the antigen to the mean background cpm obtained in the absence of the antigen.

#### ***Cytokine level determination***

Spleen cells are removed following sacrifice and splenocytes are incubated for 3 days with Ox LDL, HSP-65 or ovalbumin following which the supernatant is collected. Cytokine profile (IL-4, IL-10, IFN- $\gamma$ , and TGF- $\beta$ ) is determined at the cultured supernatant of the splenocytes.

#### **Examples**

##### **Example 1**

The mice used in this experiment are called LDL-receptor deficient (LDL-RD) mice. These animals have a genetic mutation which causes a defect in the receptor for LDL in all cells of the body. This receptor is responsible for evacuating and removing from the circulation, the 'bad' cholesterol (LDL), and when it is deficient the mice become hyperlipidemic and develop atherosclerosis when they are fed a high fat diet for 3-5 weeks.

Three groups of mice were used (15 LDL-RD mice in each group). Feeding was performed by a special device (canula) designed to be introduced to the esophagus and thus assuring the fed substances reaches mostly to the stomach.

**Group 1:** The mice were fed 5 doses of 1mg of human Ox LDL dissolved in PBS, every other day. At the end of the last dose, the mice were put on a high fat diet and sacrificed either after 3 or after 5 weeks of diet.

**Group 2:** The mice were fed 5 doses of 1 mg of a control protein (ovalbumin) in PBS and then fed with the special diet for 3 or 5 weeks.

**Group 3:** The mice were not fed prior to initiation of the diet.

The mice were evaluated, upon sacrifice for the occurrence of atherosclerotic lesions, serum cholesterol values and levels of antibodies to Ox LDL.

**Results:** All mice were of similar weight prior to, and at the termination of the study.. The mice fed OX LDL were found to develop less atherosclerosis (30% less).

In the present studies the beneficial effect of oral tolerance with Ox LDL on the induction of atherosclerosis in mice has been shown. There are many studies in which the results point to the effectiveness of oral tolerance in various autoimmune diseases, such as collagen in adjuvant arthritis (the analogue of rheumatoid arthritis), in diabetes mellitus, in uveitis, in EAE (analogue to multiple sclerosis). In the above-mentioned studies human trial was done. Recently a remarkable effect was noted when patients with rheumatoid arthritis were given per oral collagen II (oral tolerance). The comparable effect between mice studies and humans in various autoimmune diseases point to the extrapolated success in humans. It should be stressed that this treatment has no side effects.

#### **Example 2**

##### ***Suppression of high fat diet induced atherosclerosis in LDL-receptor deficient mice by feeding with human Ox LDL***

LDL-RD mice (n=15) are fed 5 doses (every other day) of human Ox LDL in three different concentrations (1mg/dose, 100ug/dose and 1mg/dose). Additional LDL-RD mice (n=15 per groups) are fed a control antigen (ovalbumin at similar doses).

One day following the last feeding, all mice are challenged with a high fat diet ('paigen') for 5 weeks.

At the end of the experiment, sera from all mice is evaluated for the presence of anti-Ox LDL, anti-HSP65 and antiphospholipid antibodies and a lipid profile is carried out (total cholesterol, LDL, VLDL, HDL and triglycerides).

Hearts from all mice are removed at the end of the study and frozen in -70°C until use (see Materials and methods) Immunohistochemical studies on the cryostat sections are performed using monoclonal antibodies to CD3, CD4, CD8, macrophages, smooth muscle cells and lymphocyte activation markers.

A similar protocol of feeding is carried out using LPC an active derivative of Ox LDL.

**Example 3*****Suppression of Mycobacterium tuberculosis (MT) induced atherosclerosis by feeding with HSP-65.***

The present inventors have observed that LDL-RD mice immunized once, with MT develop enhanced early atherosclerosis

LDL-RD mice (n=15) are fed 5 doses (every other day) of recombinant HSP65 in three different concentrations (100mg/dose, 10ug/dose and 1ug/dose). Additional LDL-RD mice (n=15 per groups) are fed a control antigen (ovalbumin at similar doses).

One day following the last feeding, all mice are challenged by an immunization with heat killed suspension of MT (10 mg/ml; 100ug/mouse) emulsified in incomplete Freund's adjuvant. The mice are sacrificed 12 weeks following the immunization with MT.

At the end of the experiment, sera from all mice is evaluated for the presence of anti-HSP65 antibodies, and a lipid profile is carried out (total cholesterol, LDL, VLDL, HDL and triglycerides).

Proliferative responses from draining lymph node cells are evaluated to HSP65 from HSP65 tolerized mice and from control fed mice.

Cytokine levels (IL-4, IFN- $\gamma$ , IL-10 and TGF- $\beta$ ) are evaluated in the supernatant collected from the medium in which lymphocytes are induced in-vitro with HSP65.

Hearts from all mice are removed and frozen in -70°C. To determine the extent of atherosclerosis cryostat sections are performed (5um per section). The relevant sections (from the aortic sinus area) are stained with the Oil-red O and the extent of atherosclerosis is determined by counting the area counted by a grid.

Immunohistochemical studies on the cryostat sections are performed using monoclonal antibodies to CD3, CD4, CD8, macrophages, smooth muscle cells and lymphocyte activation markers.

**Example 4*****Suppression of atherosclerosis by feeding with human  $\beta$ 2GPI***

The procedure is similar to the one employed for Ox LDL and HSP-65 and is based on the observation that immunization with  $\beta$ 2GPI induces early atherogenesis.

***Assessment of the extent of atherosclerosis.***

Quantification of atherosclerotic fatty streak lesions are carried out by calculating the lesion size in the aortic sinus (with a few modifications). Briefly, the heart and upper section of the aorta are removed from the animals and the peripheral fat cleaned carefully. The upper section is embedded in OCT medium and frozen. Every other section (10 $\mu$ m thick) throughout the aortic sinus (400 $\mu$ m) is taken for analysis. The distal portion of the aortic sinus is recognized by the three valve cusps which are the junctions of the aorta to the heart. Sections are evaluated for fatty streak lesions after staining with oil-red O. Lesion areas per sections are counted using a grid by an observer unfamiliar with the tested specimen.

The extent of atherosclerosis is evaluated at the level of the aortic sinus. Processing and staining of the tissue is carried out. Lesion area is quantified by the modified method of Rubin EM, Kraus RM, Spangler EA & al. (Inhibition of early atherogenesis in transegenic mice by human apolipoprotein AI. Nature 1991; 353: 265-267).

***Immunohistochemistry of atherosclerotic lesions.***

*mAbs:* Rat monoclonal antibodies *H129.19* (L3T4)-anti-mouse CD4+ and *S3-6.7* (Ly-2)-anti-mouse CD8a are obtained from PharMingen (San Diego, CA, USA) ; *MCA 497* (F4/80)-anti-mouse macrophages are obtained from Serotec (Oxford, UK).

Immunohistochemical staining for CD4, CD8 and macrophages are carried out on aortic sinus 5  $\mu$ m thick frozen sections. The sections are fixed for 4 min. in methanol at -20°C followed by 10 min. incubation with ethanol at -20°C. The sections are then blocked with non-immune goat serum for 15 min. at room temperature followed by incubation with CAS blocking reagent for 30 min. at room temperature. Subsequently, the rat-monoclonal anti-mouse CD4/CD8 is added for 1 hr at room . After washings, affinity purified biotinylated rabbit anti-rat IgG antibodies

are added for 30 min at RT. After washings, the slides are incubated with 0.3% H<sub>2</sub>O<sub>2</sub>, followed by additional rinses and incubation with streptavidin-peroxidase conjugate for 30 min at RT. After washings, the slides are developed with 3-amino-9-ethylcarbazole (AEC) substrate (Dako) for 15 min. Sections are counterstained with hematoxylin. Spleen sections are used as a positive control. Staining in the absence of 1st or 2nd antibody are used as a negative control.

***Adoptive transfer experiments***

T-cells (either CD4 or CD8 cells) will be isolated from the tolerized and non tolerized mice in each of the studies and transferred to LDL-RD littermates which will be challenged with an atherogenic diet for 6 weeks. At the end of the experiments the hearts will be taken for evaluation of atherosclerosis and immunohistochemistry.

It will be evident to those skilled in the art that the invention is not limited to the details of the foregoing illustrative examples and that the present invention may be embodied in other specific forms without departing from the essential attributes thereof, and it is therefore desired that the present embodiments and examples be considered in all respects as illustrative and not restrictive, reference being made to the appended claims, rather than to the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.



**WHAT IS CLAIMED IS:**

1. An immunological oral tolerance-inducing composition for prevention and/or treatment of atherosclerosis, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.
2. An immunological oral tolerance-inducing composition for prevention and/or treatment of a heart attack, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.
3. An immunological oral tolerance-inducing composition for prevention and/or treatment of angioplasty-restenosis, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.
4. An immunological oral tolerance-inducing composition for prevention and/or treatment of stroke, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.
5. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is a modified low-density lipoprotein.
6. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is oxidized low-density lipoprotein (Ox LDL).
7. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is an active derivative of oxidized low-density lipoprotein (Ox LDL).

8. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is heat shock protein 60/65 (HSP 60/65).
9. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is an active derivative of HSP60/65.
10. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1).
11. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is an active derivative of β<sub>2</sub>GP-1.
12. An immunological oral tolerance-inducing composition according to claim 1, wherein said active derivative is lysophosphatidyl choline (LPC).
13. An immunological oral tolerance-inducing composition according to claim 1, wherein said LDL is malondialdehyde LDL (MDA-LDL).
14. A method for prevention and/or treatment of atherosclerosis in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.
15. A method for prevention and/or treatment of a heart attack in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.
16. A method for prevention and/or treatment of angioplasty-restenosis in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1), functional

derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

17. A method for prevention and/or treatment of stroke in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

18. A method according to claim 14, wherein said active component is a modified low-density lipoprotein.

19. A method according to claim 14, wherein said active component is oxidized low-density lipoprotein (Ox LDL).

20. A method according to claim 14, wherein said active component is an active derivative of oxidized low-density lipoprotein (Ox LDL).

21. A method according to claim 14, wherein said active component is heat shock protein 60/65 (HSP 60/65).

22. A method according to claim 14, wherein said active component is an active derivative of heat shock protein 60/65 (HSP 60/65).

23. A method according to claim 14, wherein said active component is beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1).

24. A method according to claim 14, wherein said active component is an active derivative of beta<sub>2</sub>-glycoprotein-1 (β<sub>2</sub>GP-1).

25. A method according to claim 14, wherein said active derivative is lysophosphatidyl choline (LPC).

26. A method according to claim 14, wherein said LDL is malondialdehyde LDL (MDA-LDL).

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<b>(21) International Application Number:</b> PCT/IL99/00519 <b>(22) International Filing Date:</b> 30 September 1999 (30.09.99) <b>(30) Priority Data:</b> 126447 4 October 1998 (04.10.98) IL <b>(71)(72) Applicants and Inventors:</b> SHOENFELD, Yehuda [IL/IL]; Sapir Street 26, 52622 Ramat Gan (IL). HARATS, Dror [IL/IL]; Mandes Street 71, 52623 Ramat Gan (IL). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> GEORGE, Jacob [IL/IL]; Anna Frank Street 6, 49311 Petach-Tiqva (IL). <b>(74) Agent:</b> WOLFF, BREGMAN AND GOLLER; P.O. Box 1352, 91013 Jerusalem (IL).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>  <b>(88) Date of publication of the international search report:</b> 20 July 2000 (20.07.00)
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<b>(57) Abstract</b>  An immunological oral tolerance-inducing composition for prevention and/or treatment of atherosclerosis, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta <sub>2</sub> -glycoprotein-1(β <sub>2</sub> GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.		

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## INTERNATIONAL SEARCH REPORT

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## A. CLASSIFICATION OF SUBJECT MATTER

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Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GEORGE JACOB ET AL: "Induction of early atherosclerosis in LDL-receptor-deficient mice immunized with beta2-glycoprotein I." CIRCULATION SEPT. 15, 1998, vol. 98, no. 11, pages 1108-1115, XP000892137 ISSN: 0009-7322 the whole document	1-26
A	US 5 348 945 A (BERBERIAN P.A. ET AL.) 20 September 1994 (1994-09-20) cited in the application the whole document	1-26
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# INTERNATIONAL SEARCH REPORT

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1998 BONNIN DUSTAN ET AL: "Mucosal modulation of immune responses to heat shock proteins in autoimmune arthritis." Database accession no. PREV199800228934 XP002136641 abstract &amp; BIOTHERAPY (DORDRECHT) 1998, vol. 10, no. 3, 1998, pages 213-221, ISSN: 0921-299X</p>	1-26
P,X	<p>GEORGE J ET AL: "Atherosclerosis-related markers in systemic lupus erythematosus patients: The role of humoral immunity in enhanced atherogenesis." LUPUS 1999, vol. 8, no. 3, 1999, pages 220-226, XP000906739 ISSN: 0961-2033 the whole document</p>	1-26

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL 99/ 00519

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 14-26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



# INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/IL 99/00519

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5348945 A	20-09-1994	AU 659085 B	11-05-1995
		AU 7677391 A	30-10-1991
		CA 2079813 A	07-10-1991
		EP 0527783 A	24-02-1993
		WO 9115219 A	17-10-1991

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- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



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(54) Title: METHODS EMPLOYING AND COMPOSITIONS CONTAINING PLAQUE ASSOCIATED MOLECULES FOR PREVENTION AND TREATMENT OF ATHEROSCLEROSIS

(57) Abstract: Methods and compositions employing plaque associated molecules effective in inducing mucosal tolerance and inhibiting inflammatory processes contributing to atheromatous vascular disease and sequelae are provided.

METHODS EMPLOYING AND COMPOSITIONS CONTAINING  
PLAQUE ASSOCIATED MOLECULES FOR PREVENTION AND  
TREATMENT OF ATHEROSCLEROSIS

5 FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to atheromatous plaque associated molecules for prevention and treatment of atherosclerosis and related disease and, more particularly, to methods and compositions employing plaque associated molecules effective in inducing mucosal tolerance and inhibiting  
10 inflammatory processes contributing to atheromatous vascular disease and sequelae.

Cardiovascular disease is a major health risk throughout the industrialized world. Atherosclerosis, the most prevalent of cardiovascular diseases, is the principal cause of heart attack, stroke, and gangrene of the  
15 extremities, and as such, the principal cause of death in the United States. Atherosclerosis is a complex disease involving many cell types and molecular factors (for a detailed review, see Ross, 1993, Nature 362: 801-809). The process, which occurs in response to insults to the endothelium and smooth muscle cells (SMCs) of the wall of the artery, consists of the formation of  
20 fibrofatty and fibrous lesions or plaques, preceded and accompanied by inflammation. The advanced lesions of atherosclerosis may occlude the artery concerned, and result from an excessive inflammatory-fibroproliferative response to numerous different forms of insult. For example, shear stresses are thought to be responsible for the frequent occurrence of atherosclerotic  
25 plaques in regions of the circulatory system where turbulent blood flow occurs, such as branch points and irregular structures.

The first observable event in the formation of an atherosclerotic plaque occurs when inflammatory cells such as monocyte-derived macrophages adhere to the vascular endothelial layer and transmigrate through to the sub-  
30 endothelial space. Elevated plasma LDL levels lead to lipid engorgement of the vessel walls, with adjacent endothelial cells producing oxidized low

density lipoprotein (LDL). In addition, lipoprotein entrapment by the extracellular matrix leads to progressive oxidation of LDL by lipoxygenases; reactive oxygen species, peroxynitrite and/or myeloperoxidase as well as other oxidizing compounds. These oxidized forms of LDLs are then taken up in  
5 large amounts by vascular cells through scavenger receptors expressed on their surfaces.

Lipid-filled monocytes and smooth-muscle derived cells are called foam cells, and are the major constituent of the fatty streak. Interactions between foam cells and the endothelial and smooth muscle cells surrounding  
10 them produce a state of chronic local inflammation which can eventually lead to activation of endothelial cells, increased macrophage apoptosis, smooth muscle cell proliferation and migration, and the formation of a fibrous plaque (Hajjar, DP and Haberland, ME, J.Biol Chem 1997 Sep 12; 272(37):22975-78). Such plaques occlude the blood vessels concerned and thus restrict the  
15 flow of blood, resulting in ischemia, a condition characterized by a lack of oxygen supply in tissues of organs due to inadequate perfusion. When the involved arteries block the blood flow to the heart, a person is afflicted with a 'heart attack'; when the brain arteries occlude, the person experiences a stroke. When arteries to the limbs narrow, the result is severe pain, decreased physical  
20 mobility and possibly the need for amputation.

Oxidized LDL has been implicated in the pathogenesis of atherosclerosis and atherothrombosis, by its action on monocytes and smooth muscle cells, and by inducing endothelial cell apoptosis, impairing anticoagulant balance in the endothelium. Oxidized LDL also inhibits anti-  
25 atherogenic HDL-associated breakdown of oxidized phospholipids (Mertens, A and Holvoet, P, FASEB J 2001 Oct; 15(12):2073-84). This association is also supported by many studies demonstrating the presence of oxidized LDL in the plaques in various animal models of atherogenesis; the retardation of atherogenesis through inhibition of oxidation by pharmacological and/or  
30 genetic manipulations; and the promising results of some of the

interventional trials with anti-oxidant vitamins (see, for example, Witztum J and Steinberg, D, Trends Cardiovasc Med 2001 Apr-May;11(3-4):93-102 for a review of current literature). Indeed, oxidized LDL and malondialdehyde (MDA)-modified LDL have been recently proposed as accurate blood markers for 1<sup>st</sup> and 2<sup>nd</sup> stages of coronary artery disease (US Pat. Nos. 6,309,888 to 5 Holvoet et al and 6,255,070 to Witztum, et al).

Reduction of LDL oxidation and activity has been the target of a number of suggested clinical applications for treatment and prevention of cardiovascular disease. Bucala, et al (US Pat. No. 5869534) discloses methods 10 for the modulation of lipid peroxidation by reducing advanced glycosylation end product, lipid characteristic of age-, disease- and diabetes-related foam cell formation. Tang et al, at Incyte Pharmaceuticals, Inc. (US Pat. No. 5,945,308) have disclosed the identification and proposed clinical application of a Human Oxidized LDL Receptor in the treatment of cardiovascular and 15 autoimmune diseases and cancer.

Another abundant atherogenesis-related plaque component is Beta <sub>2</sub>-Glycoprotein I. Beta <sub>2</sub>-Glycoprotein I (Beta2GPI) is a 50-kDa molecule that acts as an anticoagulant in *in-vitro* assays. Although the exact role of Beta2GPI in atherogenesis has yet to be elucidated, several relevant properties 20 have been observed: 1) it is able to bind negatively charged phospholipids or phospholipid-expressing cells (apoptotic cells, activated platelets); 2) it is able to bind to modified cellular surfaces, enhancing their clearance by scavenging macrophages (Chonn A, et al J Biol Chem 1995; 270: 25845-49; and Thiagarajan P, et al Arterioscler Thromb Vasc Biol 1999; 19:2807-11); and 3) 25 it is an important target for binding of autoimmune antiphospholipid antibodies (aPLs). Beta2GPI has to undergo structural alteration in order to be recognized by aPLs. This alteration may be initiated, for example, by binding to negatively charged phospholipids or high binding plates, but also *in vivo* by binding apoptotic cells that express phosphatidylserine.

Recent studies investigating the importance of anti Beta2GPI antibodies in promoting a procoagulant state have focused on the effects of these antibodies on cellular and protein components of the coagulation system (endothelial cells, platelets and macrophages; tissue factor and coagulation factors). These studies indicate that anti Beta2GPI antibodies prevent the deactivation of platelets, sustaining their phagocytic clearance; interact with late endosomes of human endothelial cells; and suppress the inhibitory activity of the tissue factor pathway inhibitor. This association with coagulation events is consistent with Beta2GPIs proposed function in the prothrombotic antiphospholipid syndrome (APLS). Both US Pat Nos 5998223 and 5344758 (to Matsuura, et al and Krilis, et al, respectively) disclose the application of anti Beta2GPI antibodies, some to cryptic epitopes, for diagnostics in APLS and SLE. However, no therapeutic applications are disclosed by the authors.

A third important plaque-related component associated with atherogenesis is the 60/65 kDa heat shock protein (HSP). This mitochondrial protein is a member of the HSP family, which constitutes nearly 24 proteins displaying high degree of sequence homologies between different species. These proteins, as their name implies, are expressed in response to stresses including exposure to free radicals, heat, mechanical shear stress, infections and cytokines, and protect against unfolding and denaturation of cellular proteins. This has led to their designation as molecular 'chaperones'. However, HSP function may have undesired consequences, since over expression of HSPs may, under certain conditions promote an autoimmune reaction with resultant tissue damage. The mechanisms responsible for the HSP immune mediated damage are as yet unclear: it is presumed that cryptic, "non-self" neo-epitopes are exposed following their upregulation. Alternatively, it was suggested that cross-reaction exists between self-HSP and 'foreign' HSP epitopes introduced following infections which may trigger a pathological, autoimmune response against native HSP. Support for the involvement of HSP in autoimmunity is provided by studies documenting enhanced autoantibody as

well as cellular response to HSP 60/65 in several autoimmune diseases (Schoenfeld, Y et al Autoimmunity 2000 Sep; 15(2):199-202; US Pat No. 6130059 to Covacci, et al; and Gromadza G, et al Cerebrovascul Dis 2001, Oct; 12(3):235-39).

5       The link between HSP 65 and atherosclerosis was initially recognized by George Wick's group, who found that normocholesterolemic rabbits immunized with different antigens developed atherosclerosis, provided the preparation used for immunization contained complete Freund's adjuvant (CFA)(Xu, Q, et al Arterioscler Thromb 1992;12:789-99). Since the major  
10       constituent of CFA is heat killed mycobacterium tuberculosis, the principal component of which is the HSP-65, they reasoned that the immune response towards this component led to the development of atherosclerosis. This was confirmed when these authors demonstrated that immunization of animals with HSP 65 produced pronounced atherosclerosis, and that T cells from  
15       experimentally atherosclerotic rabbits overexpressed HSP-65, indicating a localized immune reaction restricted to the stressed arterial vessel. The importance of endogenous HSP-65 in atherogenesis was further demonstrated by the acceleration of fatty streak formation following HSP-65 (or Mycobacterium tuberculosis) immunization of naïve mice (George J, et al  
20       Arterioscler Thromb Vasc Biol 1999; 19:505-10;).

      Involvement of humoral immune mechanisms in response to HSP-65 were observed in atherosclerosis: a marked correlation has been found between high levels of anti-HSP65 antibodies and the extent of sonographically estimated carotid narrowing in a screen of healthy individuals  
25       (Xu Q. et al Lancet 1993; 341: 255-9; Xu Q. et al Circulation 1999; 100(11):1169-74). In addition, in-vitro experiments with cultured endothelial cells have demonstrated the concentration and time dependent induction of endothelial cell adhesion to monocytes and granulocytes following incubation with HSP65.



The association of HSP 65 with atherogenesis has led to a number of proposed therapeutic applications. Observing that immune reactivity to HSP 65 correlated with both microbial (e.g. *H. pylori*) infection and atherosclerosis, Bernie et al (Eur Heart J 1998; 19:366-7) proposed antibiotic therapy for reduction of infection and anti- HSP antibodies. Similarly, Covacci, et al (US Pat No. 6130059) disclosed the use of *H. pylori* HSPs, and related peptides, for diagnostic and therapeutic applications in atherosclerosis.

#### *Atherosclerosis and autoimmune disease*

Because of the presumed role of the excessive inflammatory-fibroproliferative response in atherosclerosis and ischemia, a growing number of researchers have attempted to define an autoimmune component of vascular injury. In autoimmune diseases the immune system recognizes and attacks normally non-antigenic body components (autoantigens), in addition to attacking invading foreign antigens. The autoimmune diseases are classified as auto- (or self-) antibody mediated or cell mediated diseases. Typical autoantibody mediated autoimmune diseases are myasthenia gravis and idiopathic thrombocytopenic purpura (ITP), while typical cell mediated diseases are Hashimoto's thyroiditis and type I (Juvenile) Diabetes.

The recognition that immune mediated processes prevail within atherosclerotic lesions stems from the consistent observation of lymphocytes and macrophages in the earliest stages, namely the fatty streaks. These lymphocytes, which include a predominant population of CD4+ cells (the remainder being CD8+ cells) were found to be more abundant than macrophages in early lesions, as compared with the more advanced lesions, in which this ratio tends to reverse. These findings posed questions as to whether they reflect a primary immune sensitization to a possible antigen or alternatively, result from previously induced local tissue damage. Regardless of the factors responsible for the recruitment of these inflammatory cells to the early plaque, they seem to exhibit an activated state manifested by concomitant expression of MHC class II HLA-DR and interleukin (IL) receptor as well as

leukocyte common antigen (CD45R0) and the very late antigen 1 (VLA-1) integrin. Thus, the inflammatory reaction of the early stages of the atherosclerotic lesion may be either the primary initiating event leading to the production of various cytokines by the local cells (i.e endothelial cells, macrophages, smooth muscle cells and inflammatory cells), or one form of the immune system's response to the hazardous process. Some of the cytokines which have been shown to be upregulated by the resident cells include TNF- $\alpha$ , IL-1, IL-2, IL-6, IL-8, IFN- $\gamma$  and monocyte chemoattractant peptide-1 (MCP-1). Platelet derived growth factor (PDGF) and insulin-like growth factor (ILGF) which are expressed by all cellular constituents within atherosclerotic plaques have also been shown to be overexpressed, thus possibly intensifying the preexisting inflammatory reaction by a co-stimulatory support in the form of a mitogenic and chemotactic factor. Recently, Uyemura et al. ( J Clin Invest 1996; 97: 2130-2138) have elucidated type 1 T-cell cytokine pattern in human atherosclerotic lesions exemplified by a strong expression of IFN- $\gamma$  but not IL-4 mRNA in comparison with normal arteries. Furthermore, IL-12 - a T-cell growth factor produced primarily by activated monocytes and a selective inducer of Th1 cytokine pattern, was found to be overexpressed within lesions as manifested by the abundance of its major heterodimer form p70 and p40 (its dominant inducible protein) mRNA.

Similar to the strong evidence for the dominance of the cellular immune system within the atherosclerotic plaque, there is also ample data supporting the involvement of the local humoral immune system. Thus, deposition of immunoglobulins and complement components has been shown in the plaques in addition to the enhanced expression of the C3b and C3Bi receptors in resident macrophages.

Valuable clues with regard to the contribution of immune mediated inflammation to the progression of atherosclerosis come from animal models. Immunocompromised mice (class I MHC deficient) tend to develop

accelerated atherosclerosis as compared with immune competent mice. Additionally, treatment of C57BL/6 mice (Emeson EE and Shen ML Am J Pathol 1993; 142: 1906-1915) and New-Zealand White rabbits (Roselaar SE , et al J Clin Invest 1995; 96: 1389-1394) with cyclosporin A, a potent  
5 suppressor of IL-2 transcription resulted in a significantly enhanced atherosclerosis under "normal" lipoprotein "burden". These latter studies may provide insight into the possible roles of the immune system in counteracting the self-perpetuating inflammatory process within the atherosclerotic plaque.

Atherosclerosis is not a classical autoimmune disease, although some of  
10 its manifestations such as the production of the plaque that obstructs the vasculature may be related to aberrant immune responsiveness. In classical autoimmune disease, one can often define very clearly the sensitizing autoantigen attacked by the immune system and the component(s) of the immune system which recognize the autoantigen (humoral, i.e. autoantibody or  
15 cellular, i.e. lymphocytes). Above all, one can show that by passive transfer of these components of the immune system the disease can be induced in healthy animals, or in the case of humans the disease may be transferred from a sick pregnant mother to her offspring. Many of the above are not prevailing in atherosclerosis. In addition, the disease definitely has common risk factors  
20 such as hypertension, diabetes, lack of physical activity, smoking and others, the disease affects elderly people and has a different genetic preponderance than in classical autoimmune diseases.

Treatment of inflammatory disease may be directed towards suppression or reversal of general and/or disease-specific immune reactivity.  
25 Thus Aiello, for example (US Pat. Nos. 6,034,102 and 6,114,395) discloses the use of estrogen-like compounds for treatment and prevention of atherosclerosis and atherosclerotic lesion progression by inhibition of inflammatory cell recruitment. Similarly, Medford et al (US Pat. No. 5,846,959) disclose methods for the prevention of formation of oxidized PUFA, for treatment of  
30 cardiovascular and non-cardiovascular inflammatory diseases mediated by the

cellular adhesion molecule VCAM-1. Furthermore, Falb (US Pat. No. 6,156,500) designates a number of cell signaling and adhesion molecules abundant in atherosclerotic plaque and disease as potential targets of anti-inflammatory therapies.

5        Since oxidized LDL, Beta2GPI and HSP 65 have been clearly implicated in the pathogenesis of atherosclerosis (see above), the contribution of these prominent plaque components to autoimmunity in atheromatous disease processes has been investigated.

*Immune responsiveness to plaque associated molecules*

10        It is known that Ox LDL is chemotactic for T-cells and monocytes. Ox LDL and its byproducts are also known to induce the expression of factors such as monocyte chemotactic factor 1, secretion of colony stimulating factor and platelet activating properties, all of which are potent growth stimulants. The active involvement of the cellular immune response in atherosclerosis has  
15        recently been substantiated (Stemme S, et al, Proc Natl Acad Sci USA 1995; 92: 3893-97), who isolated CD4<sup>+</sup> within plaques clones responding to Ox LDL as stimuli. The clones corresponding to Ox LDL (4 out of 27) produced principally interferon- $\gamma$  rather than IL-4. It remains to be seen whether the above T-cell clones represent mere contact with the cellular immune system  
20        with the inciting strong immunogen (Ox LDL) or that this reaction provides means of combating the apparently indolent atherosclerotic process.

      The data regarding the involvement of the humoral mechanisms and their meaning are much more controversial. One recent study reported increased levels of antibodies against MDA-LDL, a metabolite of LDL  
25        oxidation, in women suffering from heart disease and/or diabetes (Dotevall, et al., Clin Sci 2001 Nov; 101(5): 523-31). Other investigators have demonstrated antibodies recognizing multiple epitopes on the oxidized LDL, representing immune reactivity to the lipid and apolipoprotein components (Steineroova A, et al., Physiol Res 2001;50(2): 131-41) in atherosclerosis and  
30        other diseases, such as diabetes, renovascular syndrome, uremia, rheumatic

fever and lupus erythematosus. Several reports have associated increased levels of antibodies to Ox LDL with the progression of atherosclerosis (expressed by the degree of carotid stenosis, severity of peripheral vascular disease etc.). Most recently, Sherer et al (Cardiology 2001;95(1):20-4) demonstrated elevated levels of antibodies to cardiolipin,  $\beta$ -2GPI and oxLDL, but not phosphatidylcholine or endothelial cells in coronary heart disease. Thus, there seems to be a consensus as to the presence of anti-plaque-component antibodies in the form of immune complexes within atherosclerotic plaque.

Antibodies to Ox LDL have been implicated in both normal and pathological lipoprotein metabolism. Thus, it is known that immune complexes of Ox LDL and its corresponding antibodies are taken up more efficiently by macrophages in suspension as compared with Ox LDL. No conclusions can be drawn from this consistent finding on the pathogenesis of atherosclerosis since the question of whether the accelerated uptake of Ox LDL by the macrophages is beneficial or deleterious has not yet been resolved.

Important data as to the significance of the humoral immune system in atherogenesis comes from animal models: hyperimmunization of LDL-receptor deficient rabbits with homologous oxidized LDL, resulted in the production of high levels of anti-Ox LDL antibodies and was associated with a significant reduction in the extent of atherosclerotic lesions. Likewise, a decrease in plaque formation followed the immunization of rabbits with cholesterol rich liposomes and stimulation of production of anti-cholesterol antibodies; however, this effect was accompanied by a undesirable 35% reduction in very low density lipoprotein cholesterol levels.

Regarding the immunogenicity of Beta2GPI, it has been shown that Beta2GPI serves as a target antigen for an immune-mediated attack, influencing the progression of atherosclerosis in humans and mice. George J et al. immunized LDL-receptor deficient mice with Beta2GPI, producing a pronounced humoral immune response to human Beta2GPI, and larger early

atherosclerotic lesions in comparison with controls (George J, et al *Circulation* 1998; 15:1108-15). Afek A, et al obtained similar results in atherosclerosis-prone apolipoprotein-E-knockout mice immunized once with human Beta2GPI and fed a high fat diet for 5 weeks (Afek A et al. *Pathobiology* 1999;67:19-  
5 25).

Further, although immune reactivity to Beta2GPI in humans with the prothrombotic antiphospholipid syndrome has traditionally been attributed to the presence of autoantibodies to Beta2GPI, recent observations have indicated the importance of a cellular immune response to Beta2GPI. T-cells reactive  
10 with Beta2GPI have been demonstrated in the peripheral blood of patients with antiphospholipid syndrome. These T cells displayed a T-helper-1 phenotype (secreting the proinflammatory (and proatherogenic) cytokine interferon-) and were also capable of inducing tissue factor production (Visvanathan S, and McNiel HP. *J Immunolog* 1999; 162:6919-25). Taken together, the abundant  
15 data gathered to date regarding anti Beta2GPI (for review see Roubey RA, *Curr Opin Rheumatol* 2000; 12:374-378), indicates that the immune response to this plaque related antigen may play a significant role in influencing the size and composition of atherosclerotic plaque.

Finally, there exists a significant dependency in the antigenicity, and  
20 pathogenicity of oxidized phospholipids and Beta2GPI. As mentioned above, some of the autoimmune epitopes associated with minimally modified LDL and Beta2GPI are cryptic. Kyobashi, et al (*J Lipid Res* 2001; 42:697-709), and Koike, et al (*Ann Med* 2000; 32:Suppl I 27-31) have identified a macrophage-activating oxLDL specific ligand present only with Beta2GPI- OxLDL  
25 complex formation. This ligand was recognized by APLS-specific autoantibodies. Thus, both the pathogenic role of oxidized LDL and other plaque components, and their importance as autoantigens in atherosclerosis, as well as other diseases, have been extensively demonstrated in laboratory and clinical studies.

*Mucosal Tolerance in Treatment of Autoimmune Disease*

Recently, new methods and pharmaceutical formulations have been found that are useful for treating autoimmune diseases (and related T-cell mediated inflammatory disorders such as allograft rejection and retroviral-associated neurological disease). These treatments induce tolerance, orally or  
5 mucosally, e.g. by inhalation, using as tolerizers autoantigens, bystander antigens, or disease-suppressive fragments or analogs of autoantigens or bystander antigens. Such treatments are described, for example, in US Pat. No. 5,935,577 to Weiner et al. Autoantigens and bystander antigens are defined  
10 below (for a general review of mucosal tolerance see Nagler-Anderson, C., Crit Rev Immunol 2000;20(2):103-20). Intravenous administration of autoantigens (and fragments thereof containing immunodominant epitopic regions of their molecules) has been found to induce immune suppression through a mechanism called clonal anergy. Clonal anergy causes deactivation  
15 of only immune attack T-cells specific to a particular antigen, the result being a significant reduction in the immune response to this antigen. Thus, the autoimmune response-promoting T-cells specific to an autoantigen, once anergized, no longer proliferate in response to that antigen. This reduction in proliferation also reduces the immune reactions responsible for autoimmune  
20 disease symptoms (such as neural tissue damage that is observed in multiple sclerosis; MS). There is also evidence that oral administration of autoantigens (or immunodominant fragments) in a single dose and in substantially larger amounts than those that trigger "active suppression" may also induce tolerance through anergy (or clonal deletion).

25 A method of treatment has also been disclosed that proceeds by active suppression. Active suppression functions via a different mechanism from that of clonal anergy. This method, discussed extensively in PCT Application PCT/US93/01705, involves oral or mucosal administration of antigens specific to the tissue under autoimmune attack. These are called "bystander antigens".  
30 This treatment causes regulatory (suppressor) T-cells to be induced in the gut-

associated lymphoid tissue (GALT), or bronchial associated lymphoid tissue (BALT), or most generally, mucosa associated lymphoid tissue (MALT) (MALT includes GALT and BALT). These regulatory cells are released in the blood or lymphatic tissue and then migrate to the organ or tissue afflicted by the autoimmune disease and suppress autoimmune attack of the afflicted organ or tissue. The T-cells elicited by the bystander antigen (which recognize at least one antigenic determinant of the bystander antigen used to elicit them) are targeted to the locus of autoimmune attack where they mediate the local release of certain immunomodulatory factors and cytokines, such as transforming growth factor beta (TGF beta), interleukin-4 (IL-4), and/or interleukin-10 (IL-10). Of these, TGF-beta is an antigen-nonspecific immunosuppressive factor in that it suppresses immune attack regardless of the antigen that triggers the attack. (However, because oral or mucosal tolerization with a bystander antigen only causes the release of TGF-beta in the vicinity of autoimmune attack, no systemic immunosuppression ensues.) IL-4 and IL-10 are also antigen-nonspecific immunoregulatory cytokines. IL-4 in particular enhances (T helper 2) Th<sub>2</sub> response, i.e., acts on T-cell precursors and causes them to differentiate preferentially into Th<sub>2</sub> cells at the expense of Th<sub>1</sub> responses. IL-4 also indirectly inhibits Th<sub>1</sub> exacerbation. IL-10 is a direct inhibitor of Th<sub>1</sub> responses. After orally tolerizing mammals afflicted with autoimmune disease conditions with bystander antigens, increased levels of TGF-beta, IL-4 and IL-10 are observed at the locus of autoimmune attack (Chen, Y. et al., Science, 265:1237-1240, 1994). The bystander suppression mechanism has been confirmed by von Herreth et al., (J. Clin. Invest., 96:1324-1331, September 1996).

More recently, oral tolerance has been effectively applied in treatment of animal models of inflammatory bowel disease by feeding probiotic bacteria (Dunne, C, et al., Antonie Van Leeuwenhoek 1999 Jul-Nov;76(1-4):279-92), autoimmune glomerulonephritis by feeding glomerular basement membrane (Reynolds, J. et al., J Am Soc Nephrol 2001 Jan;12(1): 61-70) experimental



allergic encephalomyelitis (EAE, which is the equivalent of multiple sclerosis or MS), by feeding myelin basic protein (MBP), adjuvant arthritis and collagen arthritis, by feeding a subject with collagen and HSP-65, respectively. A Boston based company called Autoimmune has carried out several human  
5 experiments for preventing diabetes, multiple sclerosis, rheumatoid arthritis and uveitis. The results of the clinical trials have been less impressive than the animal experiments, however there has been some success with the prevention of arthritis.

Oral tolerance to autoantigens found in atherosclerotic plaque lesions  
10 has also been investigated. Study of the epitopes recognized by T-cells and Ig titers in clinical and experimental models of atherosclerosis indicated three candidate antigens for suppression of inflammation in atheromatous lesions: oxidized LDL, the stress-related heat shock protein HSP 65 and the cardiolipin binding protein beta 2GP1. US Patent Application 09/806,400 to Shoenfeld et  
15 al (filed Sept 30, 1999), which is incorporated herein in its entirety, discloses the reduction by approximately 30% of atherogenesis in the arteries of genetically susceptible LDL receptor deficient mice (LDL-RD) fed oxidized human LDL. Although significant inhibition of atherogenesis was achieved, presumably via oral tolerance, no identification of specific lipid antigens or  
20 immunogenic LDL components was made. Another obstacle encountered was the inherent instability of the orally fed antigen in vivo, due to digestive breakdown, and uptake of oxidized LDL by the liver and cellular immune mechanisms. It is plausible that a mucosal route of administration other than feeding (oral) would have provided tolerance of greater efficiency.

25 The induction of immune tolerance and subsequent prevention or inhibition of autoimmune inflammatory processes has been demonstrated using exposure to suppressive antigens via mucosal sites other than the gut. The membranous tissue around the eyes, the middle ear, the respiratory and other mucosa, and especially the mucosa of the nasal cavity, like the gut, are  
30 exposed to many invading as well as self- antigens and possess mechanisms

for immune reactivity. Thus, Rossi, et al (Scand J Immunol 1999 Aug;50(2):177-82) found that nasal administration of gliadin was as effective as intravenous administration in downregulating the immune response to the antigen in a mouse model of celiac disease. Similarly, nasal exposure to acetylcholine receptor antigen was more effective than oral exposure in delaying and reducing muscle weakness and specific lymphocyte proliferation in a mouse model of myasthenia gravis (Shi, FD. et al, J Immunol 1999 May 15; 162 (10): 5757-63). Therefore, immunogenic compounds intended for mucosal as well as intravenous or intraperitoneal administration should be adaptable to nasal and other membranous routes of administration.

Thus, there is clearly a need for novel methods of employing, and compositions of plaque associated molecules capable of superior tolerizing immunogenicity in mucosal, especially nasal, administration.

## SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a pharmaceutical composition for prevention and/or treatment of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis in a subject in need thereof, comprising, as an active ingredient, a therapeutically effective amount of at least an antigenic portion of at least one plaque associated molecule, or pharmaceutical salts thereof, the composition further comprising a pharmaceutically acceptable carrier, the pharmaceutical composition being designed for mucosal administration.

According to an additional aspect of the present invention there is provided an assay for determining the effect of mucosal administration of plaque components on atherosclerosis-related disease or condition, the assay effected by mucosally administering to a subject having an atherosclerosis-related disease or condition at least an antigenic portion of at least one plaque associated molecule and assessing at least one indicator of atherogenesis in the

subject to thereby determine the effect of mucosal administration of the at least an antigenic portion of the at least one plaque associated molecule on the atherosclerosis-related disease or condition.

According to yet another aspect of the present invention there is provided a method of prevention and/or treatment of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis in a subject in need thereof, the method comprising mucosally administering a therapeutically effective amount of at least an antigenic portion of at least one plaque associated molecule to the subject.

According to further features in preferred embodiments of the invention described below, the plaque associated molecule is selected from the group consisting of oxidized LDL, Beta2GPI, HSP and derivatives thereof.

According to still further features in preferred embodiments of the invention described below, the antigenic portion of at least one plaque associated molecule is a naturally occurring molecule or a synthetic molecule.

According to further features in preferred embodiments of the invention described below, the pharmaceutical composition is designed for nasal, respiratory, otic and/or conjunctival administration.

According to yet further features in preferred embodiments of the invention described below the at least an antigenic portion of the at least one plaque associated molecule is selected so as to reduce immune reactivity to plaque components in said subject.

According to further features in preferred embodiments of the invention described below, the pharmaceutical composition is packaged and identified for use in the prevention and/or treatment of at least one disorder selected from the group consisting of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis.

According to further features in preferred embodiments of the invention described below the pharmaceutical composition further comprises a therapeutically effective amount of at least one additional compound selected from the group consisting of HMGCoA reductase inhibitors (statins), mucosal adjuvants, corticosteroids, anti-inflammatory compounds, analgesics, growth factors, toxins, and additional tolerizing antigens.

The present invention successfully addresses the shortcomings of the presently known configurations by providing a novel method of inducing superior immune tolerance by mucosal administration of plaque associated molecules, thereby inhibiting atherosclerosis and other plaque related diseases.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred  
10 embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more  
15 detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1 illustrates inhibition of early atherogenesis in apo-E deficient  
20 mice by nasal tolerance induced by administration of low doses of plaque associated molecules. 9-13 week old apo-E deficient mice were exposed intranasally, with mild sedation, to 3 doses of 10 µg/mouse each HSP 65 (HSP-65)(n=12), human oxidized LDL (H-oxLDL)(n=14), human Beta2GPI (B2gpi)(n=13), bovine serum albumin (BSA) or sham exposure to saline

(PBS)(n=12). All mice received the atherogenic "Western" diet following last exposure. Atherogenesis is expressed as the area of atheromatous lesions in the aortic sinus 5 weeks following the 3<sup>rd</sup> exposure.

FIG. 2 illustrates superior inhibition of early atherogenesis in apo-E deficient mice by mucosal tolerance induced by intranasal exposure to exceedingly low doses of HSP 65. Nasal tolerance was induced in 12-16 week old apo-E deficient mice by intranasal administration of 3 doses of 1µg/mouse HSP65 (HSP-65 low)(n=16) or 10µg/mouse HSP65 (HSP-65 high)(n=14) every other day for 5 days. Control mice were exposed intranasally to an identical volume (10µl) of bovine serum albumin, 10µg/mouse (BSA)(n=14), or sham exposure to PBS (PBS)(n=14). All mice received the atherogenic "Western" diet following last exposure. Atherogenesis is expressed as the area of atheromatous lesions in the aortic sinus 5 weeks after the last nasal exposure.

FIG. 3 illustrates superior suppression of immune reactivity to atherosclerotic plaque antigens induced by nasal exposure to human Beta2GPI. 5 week old male apo-E deficient mice were exposed intranasally to 10µg/mouse human Beta2GPI (H-b2-nt)(n=3); or alternately fed, by gavage, with 100µg/mouse human Beta2GPI (H-b2-ot)(n=3) in 0.2 ml PBS; or fed PBS alone (PBS)(n=3) every other day for 5 days. One week following the last feeding the mice were sensitized with a single subcutaneous injection of 10µg/mouse human Beta2GPI in 0.1 ml volume. Ten days later T-cells from inguinal lymph node were prepared as described in Materials and Methods section that follows, and exposed to the sensitizing human Beta2GPI antigen for in-vitro assessment of proliferation. Proliferation, indicating immune reactivity, is expressed as the ratio between incorporation of labeled thymidine into the T-cell's DNA in the presence and absence of human Beta2GPI antigen (stimulation index, S.I.).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of methods and compositions employing plaque associated molecules effective in inducing mucosal tolerance to atheroma related antigens, thus inhibiting inflammatory processes contributing  
5 to atheromatous vascular disease and sequelae.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the  
10 details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

15 Experimental and clinical evidence indicates a causative role for plaque associated antigens in the etiology of the excessive inflammatory response in atherosclerosis. Both cellular and humoral immune reactivity to the plaque associated molecules oxidized LDL, Beta2GPI and HSP 65 have been demonstrated, suggesting an important anti-oxidized LDL auto-immune  
20 component in atherogenesis. Thus, oxidized LDL, Beta2GPI and HSP 65, and components thereof, have been the targets of numerous therapies for prevention and treatment of heart disease, cerebral-vascular disease and peripheral vascular disease.

Prior art teaches the application of plaque associated antigens for  
25 detection and diagnosis of atherosclerosis and other plaque- and thrombosis related conditions. For example, Holvoet (US Pat No. 6,309,888) teaches the use of stage specific plaque associated antigens oxLDL and MDA-LDL for screening for Coronary Artery disease. Similarly, others (US Pat Nos. 5,998,223 and 5,344,758 to Matsuura, et al and Krilis, et al, respectively) have  
30 disclosed the use of anti beta-2GPI antibodies to screen for serum indicators of

APLS, SLE and atherosclerosis. The abovementioned disclosures propose diagnostic applications alone, and fail to recognize the therapeutic potential of these plaque associated molecules.

Although the role of immune response in the etiology and progression  
5 of atherosclerosis and other plaque related diseases remains controversial (see Meir, K, et al, International Atherosclerosis Soc. 2001 Commentary), many immune-based therapies have been proposed for atherosclerosis. General methods of reducing immune response in inflammatory and hyperreactive conditions are taught in, for example US Patent Nos 6,277,969; 5,698,195 and  
10 5,656,272 to Le et al, and 6,224,902 to Alving, et al, International Patent Application Nos. 001 001 2514 to Shurkovitz et al and 20010051156 A1 to Zeng. However, the proposed reduction or removal of mediators of immune reactivity, such as cytokines, tumor necrosis factor (TNF) and other pathogenic factors requires ongoing costly and potentially dangerous methods such as  
15 immunoadsorption of blood and prolonged anti-cytokine administration. Furthermore, no application to treatment of atherosclerosis or plaque-related disease is disclosed.

Specific immunotherapy with plaque associated antigens has also been proposed. Bumol, et al, Calenoff, et al and Takano, et al (US Pat Nos.  
20 5,196,324; 6,025,477 and 5,110,738, respectively) disclose the use of crude, poorly defined fractionated plaque preparations for immunization, monoclonal Ab preparation, diagnosis and treatment of atherosclerosis. These antigens, protein and lipid fractions of atheromatous tissue, are poorly defined, impractical for therapeutic use, and potentially hazardous in prolonged  
25 treatment.

Prior art teaches immunotherapy directed against oxidized LDL for treatment and prevention of atherosclerosis. US Pat No. 6,225,070 to Witztum, et al discloses the use of mAb to oxidized LDL for inhibition of oxidized LDL binding to macrophages and foam cell formation. Similarly,  
30 McGregor, et al (International Patent Application EP1162458 A1) disclose

methods for specific modulation of oxidized LDL uptake and transport by macrophages. US Pat Nos 5,733,524 and 5,733,933 to Bucala, et al disclose the reduction of specific anti-oxidized LDL immune response by reduction of Advanced Glycosylation End product lipids (AGE-lipids). None of the proposed therapies teach active immunization against oxidized LDL, and require prolonged therapy regimens.

Zhou, et al (Arterioscler Thromb Vasc Biol, 2001;21:108) achieved a significant reduction in early plaque formation in mice following footpad immunization with homogenized plaque or homologous MDA LDL. Palinski et al (PNAS USA 1995;92:821-25) produced similar levels of protection in rabbits immunized with oxidized LDL. However, application of conventional immunization techniques to oxidized LDL is problematic, since the adjuvant preparations required for immunization and boosters have produced accelerated plaque formation in similar regimen of immunization. Furthermore, relatively high doses (100 µgram/ mouse/ injection) of plaque antigen were required for immunity. Mucosal administration and induction of tolerance were not mentioned.

Immune therapy with other plaque antigens has also been proposed. Recent animal and in-vitro studies with Beta2GPI (see George J, et al Rheum Dis Clin North Am 2001;27:603-10; Brey, et al Stroke 2001;32:1701-06; Kyobashi, et al J Lipid Res 2001;42:697-709; Koike T, et al Ann Med 2000;32,Suppl. I:27-31 and Cabral AR et al Am J Med 1996;101:472-81) have demonstrated the association of Beta2GPI with stroke, APLS, atherosclerosis and myocardial infarction. Although cryptic epitopes of the protein were clearly implicated in humoral and cellular immune response to oxidized LDL, none of the abovementioned studies demonstrated protective immunity with the protein. Similarly, studies with HSP 65 (Birnie DH Eur Heart J 1998;19:366-67; Xu Q, et al Circulation 1999;100:1169-74; and Gromadzka G, et al Cerebrovasc Dis 2001;12:235-39) have implicated this plaque



associated antigen in stroke and heart disease, suggesting that humoral immunity may be a triggering factor.

The complexity of plaque antigen immunity in atherosclerosis was demonstrated by Schoenfeld Y, et al (Autoimmunity 2000;15:199-202) who immunized LDL-receptor deficient (KO) mice with both HSP 65 and Beta2GPI protein antigens, producing strong cellular and humoral responses, and enhanced plaque formation. Similar increased atherogenesis was observed with passive transfer of plaque antigen activated lymphocytes. None of the above mentioned studies demonstrated inhibition of atherogenic processes by immune tolerance.

Suppression of immune response to autoantigens in atherosclerosis and related disease has been recently investigated. Victoria et al (US Pat. No. 6,207,160 and 5,844,409) discloses specific non-immunogenic Beta2GPI peptides lacking T cell epitopes for reducing antibody binding of immune cells and inducing B-cell tolerance in APLS, SLE and other diseases. However, no actual protection was demonstrated, and the disclosures emphasize the diagnostic use of the non immunogenic peptides. George J, et al (Atherosclerosis 1998;138:147-52) demonstrated the feasibility of immune suppression by hyperimmunization with MDA LDL and reduction of atherogenesis in mice. However, impractically large doses of antigen were required, and the paradoxical response to immunization with plaque antigens obviates the clinical efficacy of such therapy. Furthermore, none of the abovementioned studies disclose induction of mucosal tolerance for treatment of atherosclerosis.

Oral and mucosal tolerance for suppression and prevention of inflammatory conditions is well known in the art. Examples of candidate conditions, antigens and modes of therapy, can be found, for example in US Pat Nos. 5,935,577; 5,397,771; 4,690,683 to Weiner et al., and International Pat Nos. EP 0886471 A1 and WO 01821951 to Haas, et al. US Pat Application No 09/806,400 to Shoenfeld et al filed Sept 30, 1999, which is

incorporated herein in its entirety, teaches the oral administration of plaque associated antigens for the induction of tolerance in LDL receptor deficient mice. Measuring arterial fatty streak lesion density, the inventors demonstrated that oral administration of oxidized LDL, Beta2GPI and HSP 65 derived from animal sources were each able to produce approximately 30% reduction in atherogenesis. Typically, however, oral administration of antigens presents numerous obstacles to achieving tolerance and accurate dosing: the antigens are acted upon by digestion, altering both concentrations and molecular structure prior to their presentation to the lymphatic tissue of the Peyer's patches. Furthermore, the authors failed to investigate the efficacy of other routes of administration for induction of tolerance, such as mucosal and nasal tolerance. As the above mentioned disclosures clearly demonstrate, the parameters for induction of oral and mucosal tolerance cannot be deduced from antigenic activity in conventional immunization, or even in-vitro results, and must result from extensive empirical experimentation. Indeed, many studies have demonstrated the complexities inherent in manipulating the "balance between reacting and nonreacting" in the immune system. Zivny, et al (Clin Immunol 2001;101:150-68) clearly state that "In general, the response to one (tolerance inducing) antigen could not necessarily predict the response to another". Likewise, Hannihen et al (Diabetes 2001;50:771-75) observed that oral, nasal and respiratory administration of antigens caused appearance of disease symptoms (diabetes), rather than inducing tolerance. Similar inconsistencies in mucosal tolerance have been reported by Fujihashi et al (Acta Odontol Scand 2001;59:301-08), Jiang HR et al (Br J Ophthalmol 2001;85:739-44). Problems in mucosal vaccination strategies have been recently reviewed (Ogra PL, et al, Clin Microbiol Rev 2001;14:430-45; Chen H et al, J Control Release 2000;67:117-28; and Lehner T et al, J Infect Dis 1999;179 Suppl 3:S489-92).

While reducing the present invention to practice, the present inventors have uncovered that nasal administration of plaque associated molecules

results in the induction of mucosal tolerance, suppression of anti-plaque related antigen immune reactivity and protection from atherosclerosis. Mucosal tolerance is advantageous for its greater ease of application, accuracy of dosage and greatly reduced incidence of alteration of the tolerizing  
5 molecule by digestive and metabolic processes (especially in non-oral routes of administration). These advantages provide superior protection from atherogenic processes, improved patient compliance and reduced cost of therapy.

Thus, according to one aspect of the present invention there is provided  
10 a method of inducing immune tolerance to plaque associated molecules in a subject such as a human being.

The method, according to this aspect of the present invention is effected by administering to a subject (e.g., a human) a therapeutically effective amount of an antigenic portion of at least one plaque associated molecule.

15 As used herein, the phrase "mucosal administration" is defined as application of any and all compounds and/or compositions to mucosal membranes having component or components of the mucosal associated lymphatic tissue. Non-limiting examples of mucosal administration are buccal, intranasal, otic (middle ear), conjunctival, vaginal, rectal, etc. Mucosal  
20 administration excludes, for example, intravenous, subcutaneous and epidural administration.

As used herein, the phrase "plaque associated molecules" is defined as any and all protein, carbohydrate, lipid and nucleic acid molecules, portions thereof (antigenic portions), their derivatives, or combinations thereof  
25 physically or functionally related to the etiology, pathogenesis, symptomatology and/or treatment of a plaque related condition or disease. Such molecules may be, for example, plaque components such as oxidized LDL, foam cell components, etc, but may also include humoral and cellular entities, such as antibodies, cytokines, growth factors and T cell receptors.

As used herein, the phrase "antigenic portion" refers to a portion of a molecule capable of eliciting an immune response. For example, in cases where the molecule is a protein (e.g., HSP 65, Beta2GPI) such a portion can include a stretch of 6-8 amino acids that constitute an antigenic epitope. Methods for predicting antigenic portions are well known in the art, for example, DNASTAR'S PROTEAN sequence analysis and prediction module (DNASTar, Madison, WI). As such determining antigenic portions of plaque associated molecules suitable for use with the present invention is well within the capabilities of an ordinarily skilled artisan.

Plaque associated molecules (as well as fragments, analogs, portions and derivatives thereof) can be purified from natural sources (the tissue or organ where they normally occur) and can also be obtained using recombinant DNA technology, in bacterial, yeast, insect (e.g. baculovirus) and mammalian cells using techniques well-known to those of ordinary skill in the art. Amino acid sequences for many potential and actual plaque associated molecules are known, for example: human Beta2GPI (Accession No AAB21330 to Matsuura, et al), HSP65 (Accession No. AF65546 to Oliviera, et al) and human macrophage LDL scavenger receptor (Accession No. XP\_008489 to NCBI Annotation Project).

Immune tolerance established using the present methodology can be used in the prevention and/or treatment of disorders associated with plaque formation, including but not limited to atherosclerosis, atherosclerotic cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and in-stent-stenosis. Some non-limiting examples of atherosclerotic cardiovascular disease are myocardial infarction, coronary arterial disease, acute coronary syndromes, congestive heart failure, angina pectoris and myocardial ischemia. Some non-limiting examples of peripheral vascular disease are gangrene, diabetic vasculopathy, ischemic bowel disease, thrombosis, diabetic retinopathy and diabetic nephropathy. Non-limiting examples of cerebrovascular disease are stroke, cerebrovascular inflammation,

cerebral hemorrhage and vertebral arterial insufficiency. Stenosis is occlusive disease of the vasculature, commonly caused by atheromatous plaque and enhanced platelet activity, most critically affecting the coronary vasculature. Restenosis is the progressive re-occlusion often following reduction of occlusions in stenotic vasculature. In cases where patency of the vasculature requires the mechanical support of a stent, in-stent-stenosis may occur, re-occluding the treated vessel.

Several plaque associated molecules are suitable for use with the present method. Examples include, but are not limited to, modified lipids, phospholipids and lipoproteins, apolipoprotein-lipid complexes such as LDL-cardiolipin, specific epitopes of proteinaceous molecules such as HSP and Beta2GPI, foam cell surface antigens such as LDL receptor and smooth muscle components such as troponin.

According to a preferred embodiment of the present invention the plaque associated molecule(s) utilized by the method of the present invention is oxidized LDL, Beta2GPI, HSP 65 and/or derivatives thereof.

According to another preferred embodiment of the present invention, a combination of at least two of the abovementioned molecules is administered to the subject.

The method of the invention may be used for prevention and/or treatment of non-atherosclerosis related diseases. For example, phospholipids, phospholipid metabolites and HSP 65 have been clearly implicated in the pathogenesis, and therefore potential treatment of additional, non-atherosclerosis-related diseases. Such diseases and syndromes include oxidative stress of aging (Onorato JM, et al, Annal N Y Acad Sci 1998 Nov 20;854:277-90), rheumatoid arthritis (RA)(Paimela L, et al. Ann Rheum Dis 1996 Aug;55(8):558-9), juvenile rheumatoid arthritis (Savolainen A, et al, 1995;24(4):209-11), inflammatory bowel disease (IBD)(Sawai T, et al, Pediatr Surg Int 2001 May;17(4):269-74), renal cancer (Noguchi S, et al, Biochem Biophys Res Commun 1992 Jan 31;182(2):544-50), venous and arterial

thromboses (Cabral AR, et al Am J Med 1996;101:472-81), Anti Phospholipid Syndrome (APLS or APS) (Koike T, et al Ann Med 2000;32 Suppl I:27-31), Systemic Lupus Erythematosus (US Pat Nos. 5,344,758 and 6,207,160, to Krilis, et al and Victoria, et al, respectively). Thus, the method of the invention may be used for prevention and/or treatment of non-atherosclerosis related diseases such as aging, RA, juvenile RA, IBD, SLE, APLS, thrombosis and cancer.

The immune tolerance inducing molecules or molecule combinations described hereinabove can be administered *per se*, or in a pharmaceutical composition where they are mixed with suitable carriers or excipients.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of the pharmaceutical composition of the present invention is to facilitate mucosal administration of the immune tolerance inducing molecules to an organism.

Herein the term "active ingredient" refers to the at least an antigenic portion of the plaque associated molecules (e.g. oxidized LDL, HSP 65, and beta2GP-I) or combinations thereof which are accountable for the biological effect (immune tolerance).

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of mucosal administration may, for example, include  
5 rectal, buccal, vaginal and especially transnasal, otic, conjunctival and respiratory (including intratracheal) application.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating,  
10 emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which,  
15 can be used pharmaceutically. Proper formulation is dependent upon the mucosal route of administration chosen.

The active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer.  
20 Penetrants appropriate to the barrier to be permeated may be used in the formulation. Such penetrants are generally known in the art.

For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use  
25 of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a  
30 suitable powder base such as lactose or starch.

The pharmaceutical composition of the present invention may be administered to the membranes of the eye, such as the conjunctiva. As such, the composition may be formulated in a liquid or semi-liquid composition, as described above, for application using for example, a drop applicator. Sterility  
5 may be ensured by sterilization methods known to one skilled in the art.

The pharmaceutical composition of the present invention may be formulated in rectal and vaginal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

10 The pharmaceutical composition of the present invention may be administered by inhalation. Examples of formulations for tolerizing agents that are administered by inhalation are provided in PCT/US90/07455, filed Dec. 17, 1990. The pharmaceutical formulations for administration by inhalation of the present invention may include, as optional ingredients,  
15 pharmaceutically acceptable carriers, diluents, solubilizing and emulsifying agents, and salts of the type that are well-known in the art. Examples of such substances include normal saline solutions, such as physiologically buffered saline solutions, and water.

The route of administration of tolerizing antigens according to this  
20 alternate embodiment of the present invention is in an aerosol or inhaled form. The antigens can be administered as dry powder particles or as an atomized aqueous solution suspended in a carrier gas (e.g. air or N<sub>2</sub>). Preferred aerosol pharmaceutical formulations may comprise for example, a physiologically-acceptable buffered saline solution containing between about 1 mg and about  
25 300 mg of the antigens.

Dry aerosol in the form of finely divided solid particles of tolerizing antigens that are not dissolved or suspended in a liquid are also useful in the practice of the present invention. The tolerizing antigens may be in the form of dusting powders and comprise finely divided particles having an average  
30 particle size of between about 1 and 5 microns, preferably between 2 and 3



microns. Finely divided particles may be prepared by pulverization and screen filtration using techniques well known in the art. The particles may be administered by inhaling a predetermined quantity of the finely divided material, which can be in the form of a powder.

5        Specific non-limiting examples of the carriers and/or diluents that are useful in the by-inhalation pharmaceutical formulations include water and physiologically-acceptable buffered saline solutions such as phosphate buffered saline solutions pH 7.0-8.0. Additional non-limiting examples of suitable carriers or diluents for use in by-inhalation pharmaceutical  
10       formulations or dosage forms of the present invention are disclosed in U.S. Pat. No. 5,935,577 to Weiner, et al.

      The pharmaceutical formulations of the present invention may be administered in the form of an aerosol spray using for example, a nebulizer such as those described by Weiner, et al (US Pat No. 5,935,577). The aerosol  
15       material is inhaled by the subject to be treated.

      Other systems of aerosol delivery, such as the pressurized metered dose inhaler (MDI) and the dry powder inhaler as disclosed in Newman, S. P. in Aerosols and the Lung, Clarke, S. W. and Davia, D. eds. pp. 197-224, Butterworths, London, England, 1984, can be used when practicing the present  
20       invention. Aerosol delivery systems of the type disclosed herein are available from numerous commercial sources including Fisons Corporation (Bedford, Mass.), Schering Corp. (Kenilworth, N.J.) and American Pharmoseal Co. (Valencia, Calif.).

      Pharmaceutical compositions suitable for use in context of the present  
25       invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients effective to induce an immune response thus preventing, alleviating or ameliorating symptoms of a disorder (e.g., atherosclerosis).

Ascertaining the optimum regimen for administering the active ingredient(s) is determined in light of the information disclosed herein and well known information concerning administration of mucosally active antigens, and autoantigens. Routine variation of dosages, combinations, and duration of treatment is performed under circumstances wherein the severity of atheromatous development can be measured. Useful dosage and administration parameters are those that result in reduction in inflammatory reaction, including a decrease in number of autoreactive T-cells, or in the occurrence or severity of at least one clinical or histological symptom of the disease.

The pharmaceutical composition of the present invention can be formulated comprising a therapeutically effective amount of additional compound or compounds useful in treating or preventing plaque related disease. In one preferred embodiment the additional compounds are HMGCoA reductase inhibitors (statins), mucosal adjuvants (see, for example, US Pat No. 6,270,758 to Staats, et al), corticosteroids, anti-inflammatory compounds (see, for example US Pat No. 6,297,260 to Bandarage, et al), analgesics, growth factors, toxins and additional tolerizing agents. In addition, it will be appreciated that use of the methods and compositions of the present invention does not preclude the initiation or continuation of other therapies for the abovementioned diseases or conditions, except where of specifically counterindicated.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from *in vitro* and cell culture assays. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

In further preferred embodiments of the present invention, cytokine and non-cytokine synergists can be conjoined in the treatment to enhance the effectiveness of mucosal tolerization with plaque associated molecules. Oral and parenteral use of other cytokine synergists (Type I interferons) has been

described in PCT/US95/04120, filed Apr. 07, 1995. Administration of Th2 enhancing cytokines is described in PCT application no. PCT/US95/04512, filed Apr. 07, 1995. For example, IL-4 and IL-10 can be administered in the manner described in PCT/US95/04512.

5           Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals. The data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage  
10   form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

          Dosage amount and interval may be adjusted individually to provide  
15   mucosal levels of the active ingredient that are sufficient to induce tolerance. The "tolerizing dosage" will vary for each preparation, but can be estimated from *in vitro* data. Dosages necessary to achieve tolerizing dosage will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

20           Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

          The amount of a composition to be administered will, of course, be  
25   dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

          Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack  
30   may, for example, comprise an inhaler. The pack or inhaler may be

accompanied by instructions for administration. The pack or inhaler may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

It will be appreciated that administration of the tolerizing compositions and methods of the present invention may be effected by additional non mucosal methods such as intradermal, subcutaneous and intraperitoneal application.

According to another aspect of the present invention, there is provided an assay for determining the effects of mucosal administration of plaque associated molecules on atherosclerosis related disease or condition, the assay effected by mucosally administering the plaque associated molecule or composition thereof to a subject having such a disease or condition, and assessing at least one indicator of atherogenesis or inflammation. In a preferred embodiment, the plaque associated molecule is oxidized LDL, beta-2-GPI, HSP and/or derivatives thereof. In another embodiment at least an antigenic portion of at least one plaque associated molecule is administered mucosally, the plaque associated molecule being a naturally occurring or synthetic molecule.

Indicators of atherogenesis or inflammation that can be assessed in the context of the assay of the present invention are known to the art. Some non-limiting examples are histological methods such as fatty streak lesion count, and immunological methods such as Stimulation Index, as described herein in

Examples section that follows. Progression of atherosclerosis can be assessed, for example, in atherosclerosis-prone mice maintained on an atherogenic diet (see, for example, George J, et al Circulation 1999;99:2227-30). Inflammation can be assessed by cytological, immunological, biochemical, molecular and genetic techniques known in the art.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

### EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include biochemical and immunological techniques. Such techniques are thoroughly explained in the literature. See, for example, "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; and "Methods in Enzymology" Vol. 1-317, Academic Press; Marshak et al., all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The

procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

## 5 *Materials and Experimental Methods*

**Animals:** Apo-E deficient mice used in these experiments are from the atherosclerosis prone strain C57BL/6J-Apo-E<sup>tm1unc</sup>. Mice homozygous for the Apo-E<sup>tm1unc</sup> mutations show a marked increase in total plasma cholesterol levels which is unaffected by age or sex. Fatty streaks in the proximal aorta are  
10 found at 3 months of age. The lesions increase with age and progress to lesions with less lipid but more elongated cells, typical of a more advanced stage of pre-atherosclerotic lesion.

**Strain Development:** The Apo-E<sup>tm1unc</sup> mutant strain was developed in the laboratory of Dr. Nobuyo Maeda at University of North Carolina at Chapel  
15 Hill. The 129-derived E14Tg2a ES cell line was used. The plasmid used is designated as pNMC109 and the founder line is T-89. The C57BL/6J strain was produced by backcrossing the Apo-E<sup>tm1unc</sup> mutation 10 times to C57BL/6J mice (11,12).

The mice were maintained at the Sheba Hospital Animal Facility (Tel-  
20 Hashomer, Israel) on a 12-hour light/dark cycle, at 22-24°C and fed a normal fat diet of laboratory chow (Purina Rodent Laboratory Chow No. 5001) containing 0.027% cholesterol, approximately 4.5% total fat, and water, ad libitum. "Western diet" (TD 96125, Harlan Teklad, 42% calories from fat, 43% from carbohydrates and 15% from protein) describes a standardized, high  
25 fat atherogenic diet.

**Nasal Tolerance:** Nasal tolerance was induced by intranasal administration of oxidized LDL, Beta2GPI or HSP65, in a total volume of 10  
μl PBS. Intranasal administration was performed on mildly sedated mice (12-  
16 weeks old), each mouse receiving 3 doses of antigen per dose, in the  
30 indicated concentrations, every other day. Atherogenesis was induced by 5

weeks of a Western diet, initiated on the day following the last intranasal administration. Controls received equal amounts of BSA and/or PBS, as indicated, in an identical regimen. Plasma samples were obtained for assessment of cholesterol and triglyceride levels from all mice, and the mice  
5 were sacrificed for evaluation of atherosclerosis, as described hereinbelow, after 5 weeks Western diet.

**Oral Tolerance:** For comparison, oral tolerance to plaque associated molecules was induced by feeding 3 doses of antigen every other day (for a detailed account of induction of oral tolerance, see US Pat Application No  
10 09/806,400 to Shoenfeld et al filed Sept 30, 1999), in a similar regimen to the nasal tolerance.

#### ***Antigen Preparation***

**Beta2GPI:** Human Beta2GPI was purified from the serum of a healthy adult as described by Gharavi, et al (J Clin Invest 1992;92:1105-09).

15 **Oxidized LDL:** Human LDL (density=1.019- 1.063g/l) was prepared from Plasma of fasting individuals by preparative ultracentrifugation (50,000 rpm/min, 22 min), washing, dialysis against 150mM EDTA, pH 7.4, filtration (0.22  $\mu$ m pore size) to remove aggregation, and storage under nitrogen. LDL oxidation was performed by incubation of dialyzed, EDTA-free LDL with  
20 copper sulfate (10  $\mu$ M) for 24 hours at 37° C. Lipoprotein oxidation was confirmed by analysis of thiobarbituric acid-reactive substances (TBARS), which measures malondialdehyde (MDA) equivalents.

**HSP65:** Recombinant mycobacterial HSP-65, prepared as described (Prohaszka Z et al, Int Immunol 1999;11:1363-70) was kindly provided by Dr.  
25 M. Singh, Braunschweig, Germany.

**Immunization:** Subcutaneous immunization with human Beta2GPI: Human Beta2GPI was prepared from human plasma pool as described above. For immunization, human Beta2GPI was dissolved in PBS and mixed with equal volumes of Freund's incomplete adjuvant.  
30 Immunizations were performed by single subcutaneous injection of 10 $\mu$ g

antigen/mouse in 0.1ml volume. Three days following the last mucosal administration of plaque associated molecules the mice received one immunization, and were sacrificed 10 days post immunization.

**Cholesterol Level Determination:** At the completion of the experiment, 1-1.5 ml of blood was obtained by cardiac puncture into vials containing EDTA, centrifuged to separate plasma. Total plasma cholesterol levels were determined using an automated enzymatic technique (Boehringer Mannheim, Germany).

**FPLC Analysis:** Fast Protein Liquid Chromatography analysis of cholesterol and lipid content of lipoproteins was performed using Superose 6 HR 10/30 column (Amersham Pharmacia Biotech, Inc, Peapack, NJ) on a FPLC system (Pharmacia LKB. FRAC-200, Pharmacia, Peapack, NJ). A minimum sample volume of 300 µl (blood pooled from 3 mice was diluted 1:2 and filtered before loading) was required in the sampling vial for the automatic sampler to completely fill the 200 µl sample loop. Fractions 10-40 were collected, each fraction contained 0.5 ml. A 250 µl sample from each fraction was mixed with freshly prepared cholesterol reagent or triglyceride reagent respectively, incubated for 5 minutes at 37°C and assayed spectrophotometrically at 500nm.

**Assessment of Atherosclerosis:** Quantification of atherosclerotic fatty streak lesions was done by calculating the lesion size in the aortic sinus as previously described (George J et al Circulation 1999;99:2227-30) and by calculating the lesion size in the aorta. Briefly, after perfusion with saline Tris EDTA, the heart and the aorta were removed from the animals and the peripheral fat cleaned carefully. The upper section of the heart was embedded in OCT medium (10.24% w/w polyvinyl alcohol; 4.26% w/w polyethylene glycol; 85.50% w/w nonreactive ingredients) and frozen. Every other section (10 µm thick) throughout the aortic sinus (400 µm) was taken for analysis. The distal portion of the aortic sinus was recognized by the three valve cusps that



are the junctions of the aorta to the heart. Sections were evaluated for fatty streak lesions after staining with oil-red O. Lesion areas per section were scored on a grid by an observer counting unidentified, numbered specimens. The aorta was dissected from the heart and surrounding adventitious tissue was removed. Fixation of the aorta and Sudan staining of the vessels were performed as previously described (George J et al Circulation 1999;99:2227-30).

*Proliferation assays:* Mice were exposed to the tested antigen as described for assessment of atherosclerosis, and then immunized (one to three days following the last exposure) subcutaneously with 10 µg Beta2GPI in 0.1 ml PBS, prepared from purified human Beta2GPI as described above.

Proliferation was assayed ten days after immunization with the Beta2GPI as follows: Draining inguinal lymph nodes were prepared by meshing the tissues on 100 mesh screens. Red blood cells were lysed with cold sterile double distilled water (6ml) for 30 seconds and 2ml of NaCl 3.5% was added. Incomplete medium was added (10ml), cells were centrifuged for 7 min at 1,700 rpm, resuspended in RPMI medium and counted in a haemocytometer at 1:20 dilution (10µl cells + 190µl Trypan Blue). Proliferation was measured by the incorporation of [<sup>3</sup>H] Thymidine into DNA in triplicate samples of 100µl of the packed cells (1x10<sup>6</sup> cells/ml) in a 96 well microtiter plate. Triplicate samples of Beta2GPI (10µg/ml, 100µl/well) were added, cells incubated for 72 hours (37°C, 5% CO<sub>2</sub> and ~98% humidity) and 10µl [<sup>3</sup>H] Thymidine (0.5µCi/well) was added. After an additional day of incubation the cells were harvested and transferred to glass fiber filters using a cell harvester (Brandel) and counted using β-counter (Lumitron). Proliferation was measured by the incorporation of [<sup>3</sup>H] thymidine into DNA during the final 12 h of incubation. The results are expressed as the stimulation index (S.I.): the ratio of the mean radioactivity (cpm) of the antigen to the mean

background (cpm) obtained in the absence of the antigen. Standard deviation was always <10% of the mean cpm.

**Statistical Analysis:** A one-way ANOVA test was used to compare independent values.  $p < 0.05$  was accepted as statistically significant.

5

### EXAMPLE 1

***Inhibition of atherogenesis in genetically predisposed (Apo-E-deficient) mice by induction of nasal tolerance with low doses of the plaque associated molecules oxidized LDL, human Beta2GPI and HSP 65***

10       The present inventors here demonstrate, for the first time, that nasal exposure to low doses of the plaque associated molecules oxidized LDL, Beta2GPI and HSP 65 provides induction of tolerance to the antigens, and significant inhibition of atherogenesis. Thus, nasal exposure to purified, oxidized human LDL, human Beta2GPI and recombinant mycobacterial HSP  
15       65 were compared for their effectiveness in suppressing atherogenesis in Apo-E-deficient mice. 63 male 9-13 week old Apo E/C 57 mice were divided into 5 groups. In group A (HSP-65)(n=12) nasal tolerance was induced as described in Materials and Methods by administration of recombinant mycobacterial HSP 65 suspended in PBS (10 µg/mouse/10µl) for 5 days every  
20       other day. In group B (H-oxLDL)(n=14) nasal tolerance was induced as described in Materials and Methods by administration of 10 µg/mouse/10µl oxidized purified human LDL, suspended in PBS, every other day for 5 days. Mice in group C (B2GPI)(n=13) received 10 µg/mouse/10µl human Beta2GPI per mouse, administered intranasally as described in Materials and Methods,  
25       every other day for 5 days. Mice in group D (BSA)(n=12) received 10 µg/mouse/10µl bovine serum albumin (BSA) per mouse, administered intranasally as described in Materials and Methods, every other day for 5 days. Mice in group E (PBS)(n=12) received 10µl PBS per mouse, administered intranasally. Mice were bled prior to feeding (Time 0) and at the conclusion of

the experiment (End) for determination of lipid profile. Atherosclerosis was assessed in heart and aorta as described above, 8 weeks after the last feeding. Mice were weighed every 2 weeks during the experiment. All mice were fed water ad libitum and a normal chow-diet containing 4.5% fat by weight (0.02% cholesterol), up to the final antigen exposure, and then a "Western" diet until sacrifice.

**Table 1: Inhibition of atherogenesis in Apo-E-deficient mice by intranasal administration of exceedingly low doses of plaque associated molecules**

		HSP-65	H-oxLDL	H-B2-GPI	BSA	PBS	
Time 0	Weight (gr) (Mean±S.E)	22.6 ±0.8	22.3 ±0.5	22.3 ±0.7	21.8 ±0.7	21.7 ±0.5	P=0.833
	Chol (mg/dL) (Mean±S.E)	237 ±13	230 ±10	230 ±14	236±19	227±14	P=0.986
	TG (mg/dL) (Mean±S.E)	150 ±19	178 ±17	162 ±18	185±22	160±15	P=0.664
END	Weight (gr) (Mean±S.E)	26.8 ±0.9	28.2 ±1.0	29.2 ±1.5	25.5±1.0	26.3±1.3	P=0.157
	Chol (mg/dL) (Mean±S.E)	1181 ±114	1611 ±119	1601 ±125	1470±183	1606 ±181	P=0.197
	TG (mg/dL) (Median)	288	275	380	315	403	P=0.416
	Sinus Lesion (µm <sup>2</sup> ) (Mean±S.E)	44375 ±5437	43393 ±4107	46250 ±4486	120500 ±8746	128182 ±9102	P<0.001

Note: "Weight" is weight in grams; "Chol" is serum cholesterol and "TG" is serum triglycerides, expressed in mg/dL.

As can be seen from Figure 1, the results depicted in Table 1 demonstrate the strikingly effective inhibition of atherogenesis measured in the tissues of mice receiving nasal exposure to low doses (10 µg/ mouse) of the plaque associated molecules, compared to control mice exposed to sham antigen (BSA) or PBS. Furthermore, nasal tolerance is specific in its mode of protection: clearly, induction of nasal tolerance has no significant effect on other general parameters measured, such as weight gain, triglyceride or cholesterol blood levels. Thus, the antigenic plaque associated molecules oxidized LDL, Beta2GPI and HSP 65 are highly potent inducers of nasal

tolerance, with surprisingly low doses (10 µg/ mouse) and brief exposure (3 days) of significant (greater than 65%) and consistent protection from atherogenesis in these genetically susceptible Apo-E-deficient mice.

5

## EXAMPLE 2

### *Superior inhibition of atherogenesis in genetically predisposed (Apo-E-deficient) mice by induction of nasal tolerance with HSP 65*

The present inventors here demonstrate, for the first time, that nasal exposure to exceedingly low doses of the plaque associated molecule HSP 65 provides superior induction of tolerance to the antigen, and inhibition of atherogenesis. Thus, nasal exposure to a low dose and an exceedingly low dose of recombinant human HSP 65 were compared for their effectiveness in suppressing atherogenesis in Apo-E-deficient mice. 58 male 12-16 week old Apo E/C 57 mice were divided into 4 groups. In group A (HSP-65 high)(n=14) nasal tolerance was induced as described in Materials and Methods by intranasal administration of 10µg/mouse/10µl recombinant human HSP 65 suspended in PBS for 5 days every other day. In group B (HSP-65 low)(n=16) nasal tolerance was induced as described in Materials and Methods by administration of 1 µg/mouse/10µl recombinant human HSP 65 suspended in PBS every other day for 5 days. Mice in group C (BSA)(n=14) received 1 µg/mouse/10µl BSA per mouse, administered intranasally, every other day for 5 days. Mice in group D (PBS)(n=14) received 10µl PBS per mouse, administered intranasally. Mice were bled prior to feeding (Time 0) and at the conclusion of the experiment (End) for determination of lipid profile. Atherosclerosis was assessed in heart and aorta as described above, 8 weeks after the last feeding. Mice were weighed every 2 weeks during the experiment. All mice were fed water ad libitum and a normal chow-diet containing 4.5% fat by weight (0.02% cholesterol), up to the final antigen exposure, and then a "Western" diet until sacrifice.

**Table 2: Superior inhibition of atherogenesis in Apo-E-deficient mice by intranasal administration of human HSP 65**

		<b>HSP65 10 μg/Mouse N=12</b>	<b>HSP65 1 μg/Mouse N=16</b>	<b>BSA 100 μg/Mouse N=11</b>	<b>PBS N=10</b>	<b>Statistics</b>
End	Wt	28.4 ±1.0	26.9 ±0.9	27.7 ±0.5	28.7 ±0.7	P=0.363
	Chol	1073 ±65	1010 ±64	1009 ±74	1015 ±85	P=0.897
	TG	348 ±32	315 ±46	316 ±32	390 ±44	P=0.564
	Sinus Les. μm <sup>2</sup>	22292 ±2691	17109 ±2053	54432 ±8201	47750 ±5779	P<0.05 Between HSP- 65 and PBS or BSA

Note: "Weight" is weight in grams; "Chol" is serum cholesterol and "TG" is serum triglycerides, expressed in mg/dL.

As can be seen from Figure 2, the results depicted in Table 2 demonstrate the superior effectiveness of inhibition of atherogenesis measured in the tissues of mice receiving nasal exposure to exceedingly low doses (1 μg/ mouse) of HSP 65, compared to control mice exposed to sham antigen (BSA) or PBS. Furthermore, nasal tolerance is specific in its mode of protection: clearly, induction of nasal tolerance has no significant effect on other general parameters measured, such as weight gain, triglyceride or cholesterol blood levels. Thus, the antigenic plaque associated molecule HSP 65 is an extremely potent inducer of nasal tolerance, with even exceedingly low doses conferring significant (approximately 70%) protection from atherogenesis in genetically susceptible Apo-E-deficient mice, greatly superior to the protection achieved by induction of oral tolerance (30%; see US Patent Application No 09/806,400 to Shoenfeld et al filed Sept 30, 1999).

### EXAMPLE 3

***Superior suppression of specific anti-Beta2GPI immune reactivity in genetically predisposed (apo-E deficient) mice by intranasal administration of human Beta2GPI***

Tolerance induced by mucosal exposure to plaque associated molecules may be mediated by suppression of specific immune responses to antigenic

portions of these plaque associated molecules. Lymphocyte proliferation in response to mucosal (nasal and oral) exposure to human Beta2GPI was measured in Apo-E-deficient mice. 9 male, 5 week old Apo E/C 57 deficient mice were divided into 3 groups. In group A (n=3) oral tolerance was induced with 100 µg/mouse Beta2GPI suspended in 0.2 ml PBS, administered by gavage, as described above, every other day for 5 days. In group B (n=3) nasal tolerance was induced with 10 µg/mouse Beta2GPI suspended in 10 µl PBS, administered intranasally as described above, every other day for 5 days. The mice in group C (n=3) received oral administration of 200 µl PBS every other day for 5 days. Immune reactivity was stimulated in all mice by immunization with human Beta2GPI as described above in the Materials and Methods section, one day after the last feeding. Ten days after the immunization lymph nodes were collected for assay of proliferation. All mice were fed normal chow-diet containing 4.5% fat by weight (0.02% cholesterol) and water ad libitum.

**Table 3: Intranasal pretreatment with purified human beta2 GPI suppresses immune response to Human Beta2GPI in Apo-E-deficient mice**

	PBS	H-β <sub>2</sub> -GPI OT	H-β <sub>2</sub> -GPI NT
S.I (Stimulation Index)	7.0±0.2	4.4±0.5	2.1±0.5

As can be seen from Figure 3, the results depicted in Table 3 demonstrate significant suppression of immune reactivity to human Beta2GPI antigen, measured by inhibition of proliferation in the lymph nodes of Apo-E-deficient mice. Lymphocytes from mice receiving intranasal exposure to low atherogenesis-inhibiting doses (10 µg/ mouse) of human Beta2GPI showed an exceedingly reduced stimulation index following immunization with Beta2GPI, as compared to orally exposed and control (PBS) mice. Since previous studies with induction of nasal tolerance have shown no significant

effect on other parameters measured, such as weight gain, triglyceride or cholesterol blood levels, or immune competence (see abovementioned Examples), these results indicate a specific suppression of anti-Beta2GPI immune reactivity. Thus, intranasal administration of the purified plaque associated molecule Beta2GPI is a superior method of attenuating the cellular immune response to immunogenic and atherogenic plaque associated molecules in these genetically susceptible Apo-E-deficient mice.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents, patent applications and sequences identified by their accession numbers mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent, patent application or sequence identified by their accession number was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

## WHAT IS CLAIMED IS:

1. A pharmaceutical composition for prevention and/or treatment of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis in a subject in need thereof, comprising, as an active ingredient, a therapeutically effective amount of at least an antigenic portion of at least one plaque associated molecule, or pharmaceutical salts thereof, the composition further comprising a pharmaceutically acceptable carrier, the pharmaceutical composition being designed for mucosal administration.
2. The composition of claim 1, wherein said plaque associated molecule is selected from the group consisting of oxidized LDL, beta-2-GPI, HSP and derivatives thereof.
3. The composition of claim 1, wherein said antigenic portion of at least one plaque associated molecule is a naturally occurring molecule or a synthetic molecule.
4. The composition of claim 1, wherein the pharmaceutical composition is formulated for nasal, respiratory, otic and/or conjunctival administration.
5. The composition of claim 1, wherein said at least said antigenic portion of said at least one plaque associated molecule is selected so as to reduce immune reactivity to plaque components in the subject.
6. The composition of claim 1, packaged and identified for use in the prevention and/or treatment of at least one disorder selected from the group



consisting of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis.

7. The composition of claim 1, further comprising a therapeutically effective amount of at least one additional compound selected from the group consisting of HMGCoA reductase inhibitors (statins), mucosal adjuvants, corticosteroids, anti-inflammatory compounds, analgesics, growth factors, toxins, and additional tolerizing antigens.

8. An assay for determining the effect of mucosal administration of plaque components on atherosclerosis-related disease or condition, the assay comprising:

- (a) mucosally administering to a subject having an atherosclerosis-related disease or condition at least an antigenic portion of at least one plaque associated molecule and,
- (b) assessing at least one indicator of atherogenesis in said subject to thereby determine the effect of mucosal administration of said at least said antigenic portion of said at least one plaque associated molecule on the atherosclerosis-related disease or condition .

9. The assay of claim 8, wherein said plaque associated molecule is selected from the group consisting of oxidized LDL, beta-2-GPI, HSP and derivatives thereof.

10. The assay of claim 8, wherein said antigenic portion of said at least one plaque associated molecule is a naturally occurring molecule or a synthetic molecule.

11. A method of prevention and/or treatment of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease,

stenosis, restenosis and/or in-stent-stenosis in a subject in need thereof, the method comprising mucosally administering a therapeutically effective amount of at least an antigenic portion of at least one plaque associated molecule to the subject.

12. The method of claim 11, wherein said plaque associated molecule is selected from the group consisting of oxidized LDL, beta-2-GPI, HSP and derivatives thereof.

13. The method of claim 11, wherein said at least said antigenic portion of at least one plaque associated molecule is a naturally occurring or synthetic molecule.

14. The method of claim 11, wherein administration of said antigenic portion of said at least one plaque associated molecule is effected via nasal, respiratory, otic and/or conjunctival route.

15. The method of claim 11, wherein administration of said at least said antigenic portion of at least one plaque associated molecule reduces immune reactivity to said at least one plaque associated molecule in said subject.

16. The method of claim 11, wherein said at least said antigenic portion of said at least one plaque associated molecule is administered along with a therapeutically effective amount of a compound selected from the group consisting of HMGCoA reductase inhibitors (statins), mucosal adjuvants, corticosteroids, anti-inflammatory compounds, analgesics, growth factors, toxins, and additional tolerizing antigens.

1/2

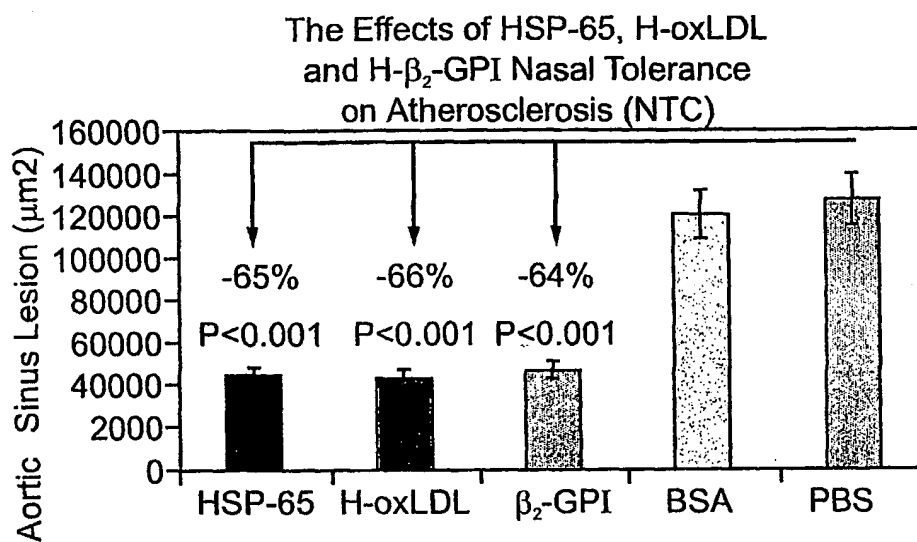


Fig. 1

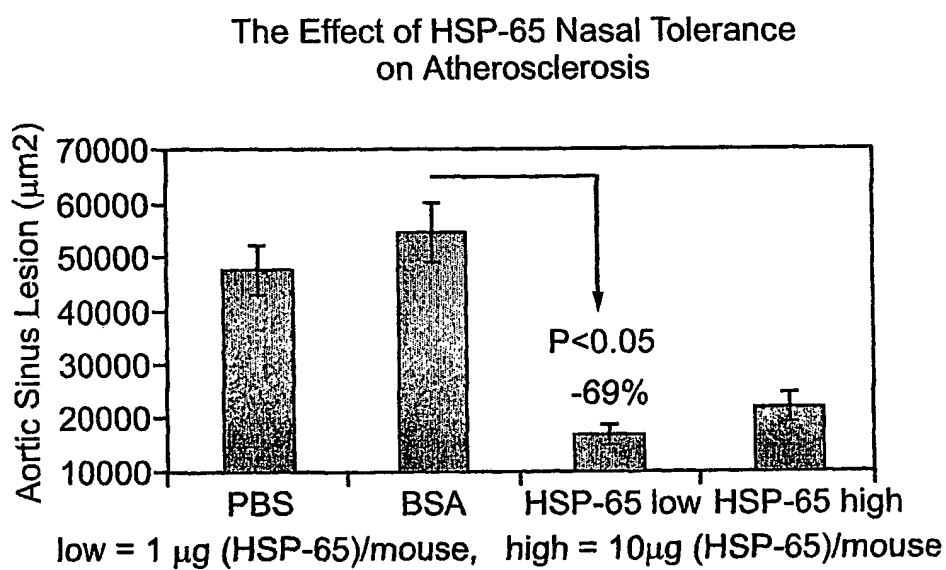


Fig. 2

2/2

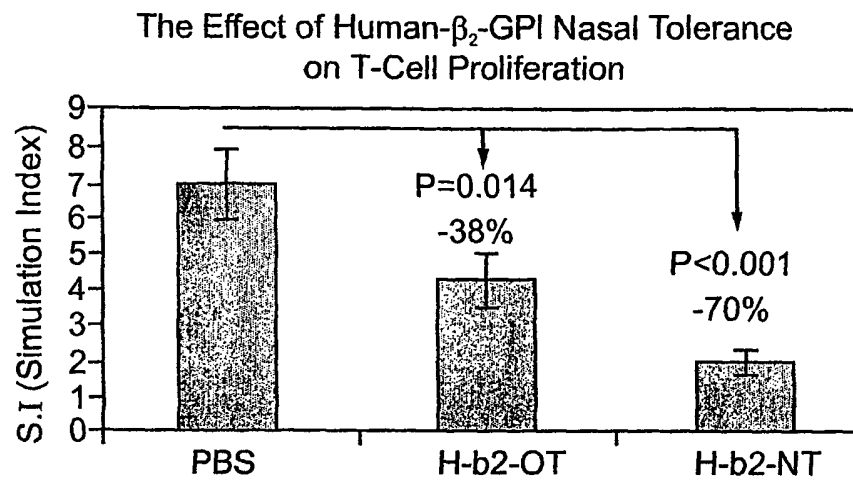


Fig. 3

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<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GEORGE et al. Hyperimmunization of apo-E-deficient mice with homologous malondialdehyde low-density lipoprotein suppresses early atherogenesis. Atherosclerosis. 1998. Vol. 138. pages 147-152, see entire document.	1-16
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
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PREVENTION AND TREATMENT OF ATHEROSCLEROSIS

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